Improved recovery and detection of *Salmonella enterica* from complex matrices

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Abstract

Salmonella enterica is the pathogen responsible for salmonellosis, a disease characterised by onset of fever, diarrhoea and nausea. The disease is commonly foodborne and has a significant burden on both the industrialised world and low-income nations. The organism is classed as a major public health concern due to the level of incidence and potential severity of infections. The majority of testing carried out for the detection of *Salmonella enterica* contamination, still heavily relies on cultural detection methods, such as enrichment and diagnostic agar. Current methodologies employ workflows that are either protracted, rely on flawed diagnostic reactions or are unable to deal with a low-level of target organism in the presence of high background and interfering matrices.

The main aims for the studies presented in this thesis were to develop and evaluate a novel chromogenic plating medium, paired with a selective single stage enrichment workflow for detection of *Salmonella enterica* from complex matrices. The study also examined the development of immunomagnetic separation (IMS) beads for the detection of *Salmonella enterica*, and specifically their use for the capture and concentration of *S.* Typhi.

The development of a novel chromogenic agar was achieved by pairing two chromogens in a highly engineered selective agar base. 5-bromo-4-chloro-3-indolyl nonanoate (X-nonanoate) was used as the target chromogen and 3,4-cyclohexenoesculetin β-D-glucoside (CHE-glc) was used as the masking chromogen. *Salmonella* possess esterase activity capable of cleaving the X-nonanoate resulting in blue green colonies on the agar surface. Several *Enterobacteriaceae* can also utilise the X-nonanoate but their activity is masked by the expression of β-D-glucosidase, which cleaves the CHE-glc resulting in black colonies. The agar was designated chromogenic agar for *Salmonella* esterase (CASE) and was shown to be superior to commercially available chromogenic media, regarding sensitivity and specificity.

The development of a single stage enrichment for *Salmonella enterica* was achieved by pairing an antibiotic cocktail with buffered peptone water (BPW). The workflow was used in conjunction with CASE to replace the standard methodology (ISO 6579) for the detection of *Salmonella enterica* from a wide range of foodstuffs. The novel workflow was compared against the standard by screening retail meat samples for *Salmonella* contamination, specifically concerned with pork mince. The prevalence of contamination for pork food products was similar to that reported previously. The alternative method reported similar results to the standard method but achieved them 24 hours earlier.

A low cost, effective IMS bead was designed and manufactured for *Salmonella enterica*. This allowed for the capture and concentration of *Salmonella* from complex matrices such as faeces, after only a six-hour resuscitation step. The produced bead was then used in a feasibility experiment, for recovering wild type *S.* Typhi isolates alongside a modified version of CASE. All isolates tested showed good reactivity with the produced IMS bead and good, typical growth on modified CASE.

The improved diagnostic agar and methodology described in this thesis allows for faster, more sensitive recovery and detection of *Salmonella enterica* from complex matrices. This provides improved tools for surveillance for this globally important pathogen.

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List of Abbreviations

EFSA – European Food Safety Authority

ECDC – European Centre for Disease Prevention and Control

PHE – Public Health England

APHA – Animal and Plant Health Agency

ISO – International Organization for Standardization

DCM – Dehydrated Culture Media

BPW – Buffered Peptone Water

XLD – Xylose Lysine Deoxycholate agar

DCA – Deoxycholate Citrate Agar

MKTTn- Muller-Kauffmann Tetrathionate-Novobiocin broth

RVS – Rappaport-Vassiliadis Soy broth

TSB – Tryptone Soy Broth

TSA – Tryptone Soy Agar

MRD – Maximum Recovery Diluent

CASE – Chromogenic Agar for *Salmonella* Esterase

OBOP - One Broth One Plate

IMS – Immunomagnetic Separation

CFU – Colony Forming Unit

MPN – Most Probable Number

SDS – Sodium Dodecyl Sulphate

NSM - Novobiocin Sulfadiazine Mannitol

CNSX – Cefsulodin Novobiocin Sulfadiazine Xylose

NCTC – National Collection of Type Cultures

ATCC – American Type Culture Collection

NTS – Non-typhoidal *Salmonella*

RpoS – RNA polymerase sigma S

List of ISO Standards Referenced

ISO 6579-1:2017
Microbiology of the food chain -- Horizontal method for the detection, enumeration and serotyping of Salmonella -- Part 1: Detection of Salmonella spp**.**

ISO 16140-2:2016
Microbiology of the food chain -- Method validation -- Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

ISO 6887-1:2017
Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 1: General rules for the preparation of the initial suspension and decimal dilutions

ISO 4833-1:2013
Microbiology of the food chain -- Horizontal method for the enumeration of microorganisms -- Part 1: Colony count at 30 degrees C by the pour plate technique

ISO 21528-1:2017
Microbiology of the food chain -- Horizontal method for the detection and enumeration of Enterobacteriaceae -- Part 1: Detection of Enterobacteriaceae

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Chapter One

Introduction

1.1 ***Salmonella***

Salmonella are Gram-negative, rod shaped, facultative anaerobic, chemotrophic, mostly motile members of the family *Enterobacteriaceae* and the causative agent of salmonellosis (Ellermeier and Slauch, 2006). This enteric pathogen takes its name from veterinary scientist Daniel E. Salmon but was characterised by Theobald Smith working under Salmon, whilst investigating swine plague. Incorrectly believing it to be the causative agent of hog cholera, the organism was described as hog cholera bacillus. The organism was in fact a common secondary infection, to the viral classical swine fever and Smith isolated what is now known as *Salmonella* Choleraesuis (Schultz, 2008). Observations had been made before Smith's work by various bacteriologists investigating outbreaks of typhoid fever, such as Eberth who isolated a bacillus from the spleen of a patient who had typhoid fever (Marineli *et al*., 2013).

The naming convention of the genus has evolved over the years resulting in confusion, especially regarding reporting the incidence of the organism. The genus is characterised by serotyping per the Kauffman-White scheme based on the somatic (O), flagellar (H) or capsular (Vi) antigen (Kauffmann, 1966, Popoff *et al*., 2000). The widely accepted consensus states that the genus now contains two species; *Salmonella bongori* and *Salmonella enterica* (Brenner *et al*., 2000). *Salmonella subterranea* is proposed as a third (Shelobolina *et al*., 2004), but recent phylogenetic analysis suggests it should be transferred to a completely different genus (Grimont and Weill, 2007). *S. bongori* is generally restricted to cold blooded animals, such as reptiles and is of usually little concern to human health (Ellermeier and Slauch, 2006) with rare reports of human infection (Pignato *et al*., 1998). *S. enterica* consists of six subspecies of which *S. enterica* subsp. *enterica* contains many of the serovars of significance (Chan *et al*., 2003). Currently there are over 2500 recognised serovars of *S. enterica* (Popoff *et al*., 2004), which can be broadly grouped into two categories: typhoidal and non-typhoidal. Both groups have high burdens of disease and despite their genetic similarity, result in very different diseases. Typhoidal *Salmonella* include *S. enterica* serovars *S.* Typhi and *S.* Paratyphi A, B & C, which are responsible for typhoid and paratyphoid fever, respectively. There are other serovars that can

cause enteric fever (the general name for disease caused by typhoidal *Salmonella),* such as *S.* Sendai (Taylor and Eves, 1969), however Typhi and Paratyphi are the most common. Typhoidal *Salmonella* are invasive, cause life threatening disease and tend to have a long incubation period (up to 21 days), with symptoms lasting up to three weeks and can result in individuals becoming long term carriers, but are restricted to the human host which are their only reservoir (Gal-Mor *et al*., 2014). Non-typhoidal *Salmonella* (NTS) such as *S.* Typhimurium and *S.* Enteritidis are generalist pathogens and a leading cause of gastroenteritis (EFSA and ECDC, 2017). Whilst many *Salmonella enterica* serovars are generalist pathogens, there are some serovars that are more adapted to infection of specific hosts. As previously mentioned *S.* Typhi is a human host adapted serovar, whose virulence factors are well studied (Kaur and Jain, 2012). However, the virulence factors of the other host adapted serovars are less understood. *S.* Dublin is associated with the majority of *Salmonella* infections in cattle (Kingsley and Bäumler, 2000) and is adapted to its bovine host. Whilst *S.* Dublin is associated with cattle it is also associated with rare but serious invasive and systematic disease in humans (Mohammed *et al*., 2017). Invasive refers to the disease progressing to the bloodstream or organs of the body, resulting in a much more life-threatening disease. Another example of a specialist serovar is *S.* Gallinarum, which is an avian host adapted serovar with devastating effects on the health of chicken flocks (Shivaprasad, 2000). Non-typhoidal *Salmonella* infections cause a wide range of symptoms, such as fever and diarrhoea but are generally self-limiting with only around 5% developing bacteraemia and further complications (Acheson and Hohmann, 2001). Non-typhoidal *Salmonella* tend to have a short incubation period (<24 hours), with symptoms lasting less than ten days and have a wide range of reservoirs including livestock and pets and can be found on fresh produce (Gal-Mor *et al*., 2014). Whilst NTS infection generally presents as an unpleasant self-limiting bout of gastroenteritis it has also now become a major cause of serious disease amongst immunocompromised individuals, such as those with HIV (Feasey *et al*., 2012). Typhoidal *Salmonella* and the resulting enteric fever causes greater than 25 million of incidence of disease and over 200,000 deaths per year, with the majority of these mostly restricted to Africa and also Asia (Crump *et al*., 2004). In comparison, gastroenteritis

caused by NTS results in nearly 100 million cases with around 150,000 deaths per year globally (Majowicz *et al*., 2010). Due to a very low level of typhoidal salmonellosis cases originating in Europe and the UK, *Salmonella enterica* is largely considered in Europe by its impact on food and food production and resulting litigation. According to the Health Protection (Notification) Regulations 2010, Enteric fever (typhoid or paratyphoid fever) is a notifiable disease in humans in the UK. This means that the relevant government body must be alerted to the incidence of disease by law. *Salmonella* spp. are listed as a causative agent that must be notifiable to Public Health England (PHE). *Salmonella* is also classed as a reportable disease in animals in the UK. This means if a laboratory finds evidence of infection/disease it must be reported to the Animal and Plant Health Agency (APHA). Also, due to the Regulation (EC) No. 2160/2003 *Salmonella* is subject to surveillance and controlled via national control programs. The European Food Standard Agency (EFSA) estimates the annual cost of salmonellosis in humans is approximately €3 billion (EFSA, 2014a). In low income countries typhoidal salmonellosis is endemic due to poor sanitation and water management, meaning the disease can spread via the faecal-oral route. This is the main reason why typhoidal *Salmonella* are rare in the developed world since clean water and adequate sanitation is widely available. These two groups of *Salmonella* embody the two different focuses of management of the organism, firstly NTS as a foodborne pathogen of great concern to middle to higher income counties and secondly typhoidal *Salmonella* as a serious and often deadly cause of disease in lower income countries. However, this is an over simplification as NTS also has a high impact on low income countries, arguably more so than the higher income countries.

Historically one of the most common sources associated with foodborne salmonellosis was contaminated hens eggs (Gantois *et al*., 2009). However, great effort has been put into curbing zoonotic infections by *Salmonella* via vaccination of egg laying hens and increased biosecurity, which have generally been successful (Kilroy *et al*., 2016). Foodborne outbreaks associated with *Salmonella* have been reported from a wide array of foodstuffs, such as bean sprouts (Cleary *et al*., 2010), lettuce (Gajraj *et al*., 2012), frozen meat (Huusko *et al*., 2017) and pork products (Schroeder *et al*., 2016).

Dissemination of *Salmonella* in the environment and its ability to survive in the food chain make it one of the greatest challenges for food safety (Humphrey, 2004). Due to successful control programs the current advice on eggs (that carry the red British lion stamp) in the UK is that they can now be eaten raw, due to a massive reduction in prevalence of *Salmonella*. However, due to the diversity and ability to adapt, the pathogen has become more problematic in other reservoirs. Currently pig meat products are responsible for an estimated 20% of human *Salmonella* infections in Europe (EFSA, 2010), and is partly responsible for the troubling rise of monophasic variants in the UK (Mueller-Doblies *et al*., 2013).

1.2 Microbial detection

The core principle of microbial detection from food and environmental samples, is that the target organism if present, will be at too low a level to reliably detect using traditional methods. Microbiological culture media provides an environment which allows the organism to reproduce to levels favourable for detection. Most protocols involve some sort of enrichment followed by a diagnostic test. There are however limitations of microbial detection, which arise from four major problems; the complexity of the food matrices being tested, the uneven distribution of a organisms in the matrices, the physiological state of the organism and the presence of competing non-target organisms (López-Campos *et al*., 2012).

The matrix itself can have a large impact on the result of the test. The matrix may greatly alter the environment of the primary enrichment due to the nature of its own composition, for example coleslaw may shift the pH due to its acidity, or milk powder may increase the nutritional availability due to its casein content. This effect can be detrimental to the detection of a target analyte. The matrix may also contain inhibitor compounds that can interfere with the testing platform and result in a false negative (Schrader *et al*., 2012). Examples include proteases in milk products (Powell *et al*., 1994) and complex polysaccharides in faeces (Monteiro *et al*., 1997). Testing laboratories often undergo proficiency testing to routinely evaluate the suitability and performance of their testing regime. Such

schemes involve samples submitted to laboratory by an external examining body for testing which have known contamination status. Reported results are compared to the actual results to judge if a testing protocol is performing adequately. This however, is not an absolute test of the interfering effect of sample matrix due to the nature of the reference materials used (Abdelmassih *et al*., 2014).

When testing for pathogens in foodstuffs a portion of the material will be used as a representative sample. This can be an issue depending on the distribution and level of the potential microbial contamination in the batch (Jongenburger *et al*., 2015). If the sample selected for testing happens to be absent of the target analyte, it does not necessarily mean that the analyte is absent in the entire batch of foodstuff.

In an effort to reduce microbial contamination, control measures are often implemented in food processing, which can include treatments, such as those involving extremes of temperature, pH, salinity and control of humidity (Jay, 2012). These control measures may not effectively kill pathogens, but rather damage them so that their ability divide and multiply is reduced. When enrichment in culture media is attempted, there may be an extended lag phase which can result in lower final numbers and therefore a lower probability of detection. In more extreme cases this could result in the organism entering a state where it is unable to multiply in traditional culture media. This state is known as viable but non-culturable (VBNC) and was demonstrated *in vitro* by Xu *et al.* over thirty years ago (Xu *et al*., 1982). These injured microorganisms are still an issue for food safety, since despite the failure to initially grow in culture media, they may recover at a later stage in the food chain possibly resulting in infection (Wu, 2008). The terminology of non-culturable is confusing since these cells may be culturable under the right conditions, but just fail to grow and give a diagnostic result with traditional culture media (Oliver, 2000). There is great debate of the significance of the VBNC state for food safety testing. There is a lack of evidence of the phenomenon occurring outside of the research laboratory and causing a detection failure, leading to infection. However, it is widely accepted that the phenomenon is poorly understood and further research is needed (Ramamurthy *et al*., 2014).

One of the most common issues in detecting a pathogen in a sample is the presence and effect of high levels of non-target organisms. In the first instance, the background flora may compete with the target organism during the enrichment stage and later, on the solid media non-target organisms may physically obscure the isolation and identification by over growing on the agar surface.

Sample matrices are often contaminated with mixed populations of microorganisms. Growth of a mixed culture can be detrimental to the recovery and detection of a single member of the population. The negative effect of the food microbiota on the growth kinetics of *Salmonella* has been demonstrated in food matrices (Zaher and Fujikawa, 2011) and in culture media (Jameson, 1962). Jameson (1962) elaborated on the dynamics of a mixed culture enrichment of *Salmonella*, stating that in a culture medium with a mixed population where one organism is in the minority, both will grow rapidly until one has reached a near molar concentration. At which point both will end their rapid growth phase. The organism in the minority prematurely enters the stationary phase, resulting in much lower numbers compared to that of it in pure culture under the same conditions. This limit of growth has been described as “metabolic crowding” where there is a limit to the number of active metabolising cells a medium can support (Schiemann and Olson, 1984). The negative effect on productivity of *Salmonella* pre-enrichment by Gram-negative organisms results in an unfavourable ratio of microorganisms for *Salmonella* detection. The negative effect is not due to nutrient limitation, toxic metabolic by-products or non-optimal pH (Abbiss, 1986, Davis, 1991), but is strongly linked to the redox potential (Eh) of the media regulated by RpoS (a gene which encodes proteins which regulate transcription) induction (Komitopoulou *et al*., 2004). These findings suggest it is low redox potential and not oxygen that causes the inhibition of *Salmonella* growth. It is hypothesised that the sensing of the redox potential and the effect it has on RpoS is due to redox sensitive signalling molecules of Gram-negative organisms. This competitive effect that can result in the failure of microorganisms to grow to detectable levels is known as the Jameson effect. The Jameson effect is only observed when both organisms are Gram-negative. Competitive restrictions are not seen between Gram-positive and Gram-negative populations, as Gram-positive organisms do not cause a

significant drop in the redox potential when they reach stationary phase (Komitopoulou *et al*., 2004). It is one of the main reasons selective secondary enrichment is required since the selective pressure will favour the target organism and prevent the non-target background from exceeding the numbers of the target organism. However, this is not always the case, especially for *Salmonella* detection. Even after a selective enrichment step, levels of non-target organisms can exceed those of *Salmonella*. This is due to the relatedness of similar *Enterobacteriaceae* and such, competitive microorganisms often have the same growth requirements and selective resistance of *Salmonella.* A good example is *Citrobacter* species which can grow as well in some cases in selective culture media formulations for *Salmonella,* due to its ability to tolerate the selective pressures employed. So, if the enriched sample is subcultured onto an agar plate it is possible that the agar could be overcrowded with non-target organisms which may obscure the visual identification of any *Salmonella* colonies that may be present.

1.3 Cultural methods and media

No cultural method is capable of always recovering and detecting *Salmonella* spp. This is mainly due to the diversity of this genus in terms of optimal growth conditions and sensitivity. In fact the result of a given test is greatly affected by the culture media used, especially the selective agents employed (Love and Rostagno, 2008). The development of the culture media used today is a story of continued modification and improvement for over 100 years, with researchers building on the discoveries of their predecessors.

Edel and Kampelmacher first pioneered the routine use of a buffered peptone medium for *Salmonella* enrichment methods, now widely known as buffered peptone water (BPW) (Edel and Kampelmacher, 1973). Later studies showed that BPW was superior to the commonly used lactose broth due to its lower nutritional content, reducing the risk of over growth of competitive organisms *(*Thomason *et al*., 1977). In the late 70's and onwards it became evident that pre-enrichment in BPW was key to reviving sub-lethally damaged *Salmonella* to ensure growth in the subsequent selective systems (Siems, 1974). BPW is a relatively simple media that contains a peptone source (normally from casein),

sodium chloride to maintain the osmotic balance and a phosphate buffer system to maintain the pH. The peptone provides a source of nitrogen, carbon and other compounds essential for growth. The osmotic pressure generated by the sodium chloride creates a favourable environment and improves cell integrity. The buffered system prevents unfavourable pH shifts that could be caused by metabolic activity or the sample matrices. Damaged organisms are sensitive to extremes of pH meaning that left unbuffered the culture media environment could be unfavourable to growth. Since its widespread use from the 1980's modifications have been proposed to improve its performance. Researchers have added selective components, such as the antibiotic, novobiocin (Jensen *et al*., 2003) or bile acids (Margot *et al*., 2015) and elective components (non-nutritious growth promoting compounds), such as the siderophore, Ferrioxaimine E (Reissbrodt *et al*., 1996). It has also been shown that the peptone composition has a highly significant effect on the generation time and yield of *Salmonella* (Gray *et al*., 2008). Regardless of the modification, none have gained the same widespread use as the standard formulation of BPW. This is either due to cost or negligible benefits when implemented with various sample types and bioburden levels.

After pre-enrichment many protocols for *Salmonella* detection involve a selective enrichment step, since pre-enrichment will most likely also result in high levels of non-target organisms. The secondary enrichment is no longer concerned with resuscitation, but instead suppressing or inhibiting those organisms that would interfere with the final diagnostic test. It also allows for further multiplication of the target organism increasing its chance of detection. Like the primary stage the secondary enrichment media also provides a nutritious base for growth, but now contains selective compounds that favour the growth of the target organism over other competitive microorganisms. The use of bile in selective media for enteric organisms can be largely credited to Alfred Theodore MacConkey, who at the turn of the 20th century whilst working with colleagues at the University of Liverpool developed several media employing various bile acid fractions (MacConkey, 1908). Bile acids, specifically secondary bile acids like deoxycholate are membrane active and are effective at inhibiting Gram-positive organisms in culture media formulations. However, bile acids also favour growth of other

Enterobacteriaceae. Researchers started to also formulate media containing the dye brilliant green to improve selectivity (Dunham and Schoenlein, 1926), since brilliant green was able further inhibit competitive microorganisms. The use of aniline dyes such as brilliant green and others had been known previously (Krumwiede *et al*., 1916), but was not well defined enough to be properly exploited in culture media until the early 1920's. Work by Muller (Muller, 1923) showed the potential of using tetrathionate as a selective agent. In his broth, Muller observed that lactose fermenting *Enterobacteriaceae* were inhibited or suppressed whilst *Salmonella* grew well. Kauffmann later modified the formulation to include ox bile and brilliant green to improve selectivity (Kauffmann, 1935). Finally, Jeffries added Novobiocin to inhibit *Proteus* species (Jeffries, 1959). The modern widely used version is called Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn).

Figure 1.1 The appearance of Muller-Kauffmann Tetrathionate-Novobiocin broth, 10 ml in a plastic universal. The media is opaque when prepared due to the high level of calcium carbonate in the formulation present to buffer the sulphuric acid generated from tetrathionate reduction.

Tetrathionate is generated by the addition of iodine-iodide solution which causes oxidation of the thiosulphate present. This is favourable to adding the pure compound since tetrathionate is significantly more expensive than the generating system. MKTTn works well, however it has

limitations since some *Salmonella* are sensitive to brilliant green and grow poorly or fail to grow at all and in some cases, it can allow for relatively high growth of non-target organisms. Noting the problems associated with the Muller-Kauffmann broth, a new broth was developed using a different selective system known as Rappaport broth (Rappaport *et al*., 1956). This broth no longer utilised ox bile, but relied upon high osmotic pressure from magnesium chloride (MgCl) and the dye malachite green for selectivity. Vassiliadis later increased the incubation temperature to 43°C from 37°C and the concentration of malachite green was reduced (Vassiliadis *et al*., 1976). This improved both the selectivity and sensitivity of the broth which became known as Rappaport-Vassiliadis broth (RV). Studies showed that RV performed better than the original Muller-Kauffmann formulation for the recovery of *Salmonella* from samples with high non-target background (Vassiliadis, 1983). Finally efforts by van Schothorst *et al*. (1987) to enhance the reliability of the broth led to a titration of the level of MgCl, a change of the peptone to a soya source rather than the original tryptone and buffering of the medium (van Schothorst *et al*., 1987).

Figure 1.2 The appearance of Rappaport-Vassiliadis Soya peptone broth when prepared (right) and after the growth of *Salmonella* (left).

This formulation became the modern widely used broth known as Rappaport-Vassiliadis Soya peptone broth (RVS). The broth is favourable for growth of *Salmonella* due to their ability to better tolerate

low pH, high osmotic pressure and presence of malachite green compared to other common food contaminants such as *E. coli* and *Proteus* spp. Rappaport-Vassiliadis medium was also taken as a base to create a semisolid motility medium called modified semisolid Rappaport-Vassiliadis (MSRV) medium (De Smedt *et al*., 1986). The medium works on the principle that most *Salmonella* are motile and migrate through the medium faster than other motile organisms that are also resistant to the selective agents present. A small amount of agar is incorporated to make it semisolid but not completely set and thus allowing for migration of motile organisms. The migration produces opaque halos of growth from the original inoculation point. MSRV has been shown to be particularly useful for *Salmonella* detection from certain matrices, such as chocolate (De Smedt *et al*., 1994, De Smedt *et al*., 1991).

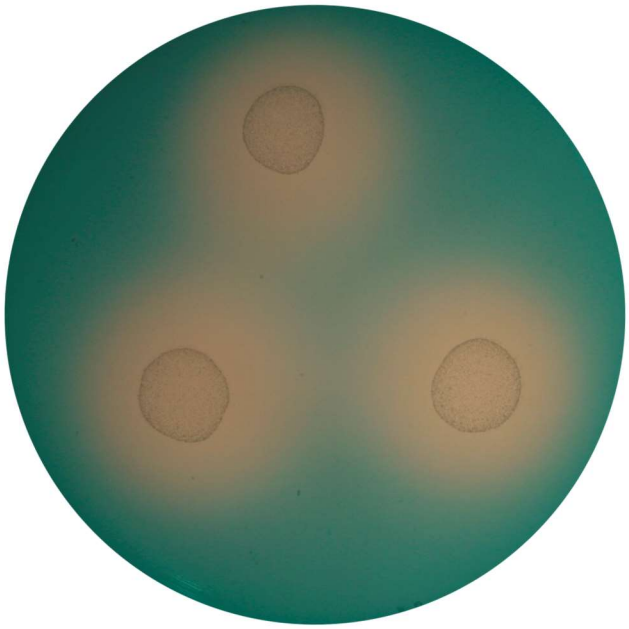


Figure 1.3 The appearance of *Salmonella* growing on modified semisolid Rappaport-Vassiliadis medium. Opaque halos can be seen around the three inoculation points on the agar, indicating motility.

After growth in liquid culture a small volume (typically 5-10µL) of the enrichment broth is streaked onto an agar plate to visualise a single colony forming unit (CFU). Agar media for *Salmonella* employ

the same types of selective agents as those found in liquid media, but also employ diagnostic systems to help identify *Salmonella*. They typically are based on a set of biochemical rules which generally apply to *Salmonella.* Those rules include the inability to ferment lactose and sucrose, decarboxylate lysine and produce hydrogen sulphide (H_2S) from thiosulphate and other sulphur sources. The most widely used plating media for *Salmonella* is xylose lysine deoxycholate (XLD) and was originally developed by Taylor (Taylor, 1965) for an improved detection of *Shigellae* from stools. The diagnostic system works by detecting the H_2S production of *Salmonella* species which in combination with the ferric ammonium citrate (FAC) yields black colonies.

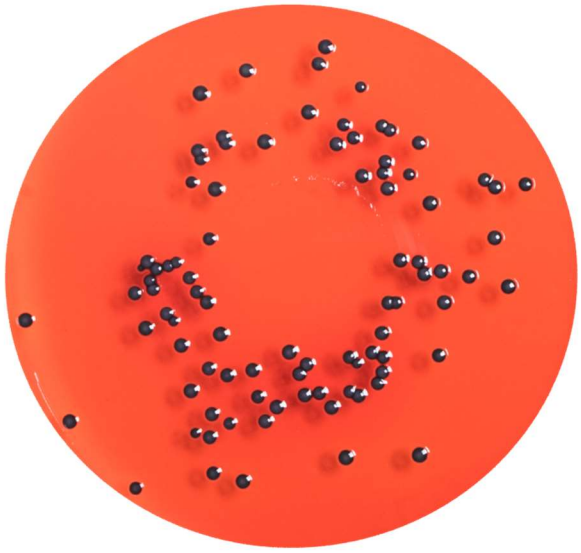


Figure 1.4 The typical appearance of *Salmonella* on xylose lysine deoxycholate agar (Lab M). *Salmonella* grow as clear colonies with black centres.

Xylose is included since most enteric organisms except *Shigella* species will ferment this carbohydrate to produce acid and lower the pH. *Salmonella* only reduces the thiosulphate at near neutral pH so the amino acid L-Lysine is included, which *Salmonella* will decarboxylate and shift the pH towards neutral. Other organisms that are also able to produce H_2S are prevented by the inclusion of lactose and sucrose. Organisms such as *Citrobacter* will ferment these carbohydrates and produce acid, which prevents the ability to reduce the thiosulphate. Sodium deoxycholate is included as a selective agent

to inhibit or suppress other *Enterobacteriaceae,* such as *E. coli*. There are many other agars for the detection and identification of *Salmonella,* such as Brilliant Green Agar (BGA), Deoxycholate Citrate Agar (DCA), Bismuth Sulphite Agar (BSA), Xylose Lysine Tergitol-4 Agar (XLT4) and Hektoen Enteric Agar.

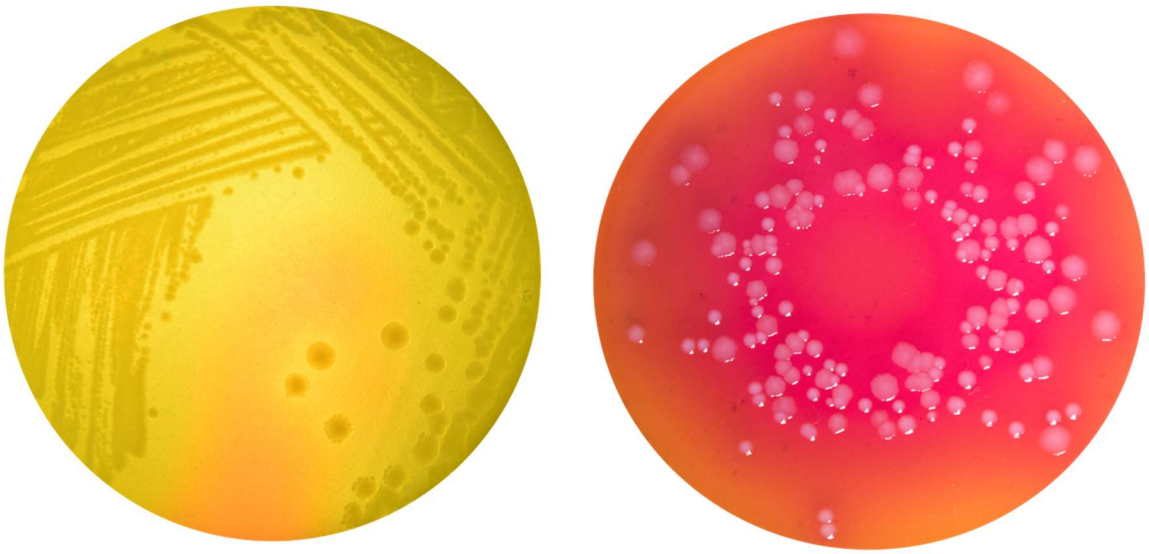


Figure 1.5 The appearance of *Salmonella* on brilliant green agar (right) and the appearance of a lactose fermenting, brilliant green tolerant *Enterobacteriaceae* (left). *Salmonella* cannot ferment lactose so do not produce acid and drop the pH the media, thus grows clear or red. Organisms that ferment lactose will produce acid, which is visualised by a pH indicator dye causing the agar to turn yellow.

These agars have similar diagnostic features and rely on the same biochemical rules as previously mentioned. However, due to the diversity of *Salmonella* spp. and bacteria in general there are many isolates that have been identified that do not follow these rules and are atypical. Lactose fermenting *Salmonella* have been reported for many years (Kunz and Ewing, 1965, Gonzalez, 1966), as have *Salmonella* that do not produce H_2S (Adám and Kádár, 1982, Aksoysan *et al*., 1981, Copeland *et al*., 1956). These atypical isolates are an issue for traditional diagnostic tests like XLD agar as they will produce a false negative result and can go undetected.

1.4 ISO 6579

ISO 6579 specifies a horizontal method for the detection of *Salmonella* (ISO, 2017). Cultured based methods such as those described by ISO 6579, or other very similar protocols are the most widely used and remain the gold standard in for *Salmonella* detection worldwide (Odumeru and Leon-Velarde, 2012). ISO 6579 was developed by the International (ISO) and European Standardisation (CEN) Organisations and is reviewed typically every 5 years (Mooijman, 2018). The first issue was in 1981 and was a general guidance document for the detection of *Salmonella*. The current edition published in 2017 is titled; "Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella*". This document is split into three separate parts. The first and main part describes the advised detection methods, the second describes methodology for enumeration by a miniaturised Most Probable Number (MPN) method and the third describes the serotyping methodologies for *Salmonella*. The MPN method allows for the enumeration of *Salmonella* in samples where knowing the level of contamination would be beneficial, such as boot socks from an animal enclosure. Multiple dilutions of the sample are tested and counted so that the level of contamination in the original sample can be estimated. ISO 6579 is applicable to products intended for human consumption and the feeding of animals, environmental samples in the area of the food production and food handling and samples from the primary production stage such as animal faeces, dust and swabs. The standard contains all the recommended culture media formulations that are included in the prescribed method. The concept is that users can source the raw materials and prepare the culture media as described by following the instructions. However, due to the lack of expertise in this area and convenience, most users purchase commercially available products, formulated and tested according to the relevant standards. The ISO standards refer to this, thus the culture media descriptions are mainly used to compare the commercial product technical specifications against.

The testing methodologies are separated into three protocols, but all have a multistage approach that uses several enrichment processes. The first is a procedure for the detection of *Salmonella* in food,

animal feed and environmental samples from the food production area. This procedure (displayed in Appendix 6) starts with a tenfold dilution of sample matrices into BPW, which is then incubated for 18 h ± 2 h at 34°C to 38°C. From this enrichment, a 0.1mL subculture is taken into RVS broth or MSRV and incubated for 24 h ± 3 h at 41.5°C ± 1°C. At the same time 1mL is subcultured into MKTTn broth and incubated for 24 h ± 3 h at 37°C ± 1°C. Both these secondary enrichments are then individually streaked onto XLD agar and a second isolation agar of choice. XLD is incubated for 24 h ± 3 h at 37°C ± 1°C. Any typical or suspect colonies are taken for further biochemical and serological testing after subculture and growth on non-selective agar. The second protocol is for the detection of *Salmonella* in animal faeces and in environmental samples from the primary production stage. This protocol is identical to the first, but secondary selective enrichment is replaced by a duplicate enrichment in MSRV only. The final protocol prescribes the detection of *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi. The protocol is identical to the first except for the addition of selenite cystine medium alongside RVS and MKTTn broth and the addition of bismuth sulphite agar alongside XLD.

1.5 Capture and concentration of microorganisms by immunomagnetic separation (IMS)

In most samples, the presence of *Salmonella* is almost always accompanied by a much larger level of non-target bacteria. The ratio of microorganisms being much more in the favour of the non-target, means that diagnostic tests may struggle to detect the low level of the target. Immunomagnetic separation (IMS) is a method that can be used before or after enrichment, to improve the sensitivity and specificity of a diagnostic test such as differential agars. IMS uses superparamagnetic (only magnetized when under a magnetic field) particles coated with a specific ligand, which is able to bind to a target in a heterogeneous target suspension (Yakub and Stadterman-Knauer, 2004). The technology can capture and concentrate a target analyte in the presence of high background non-target. The technology has found application in various aspects of life sciences, including capture and concentration of DNA, RNA and proteins (Lundeberg and Larsen, 1995) and tumor cells (Clarke and Davies, 2001). In microbiology, IMS technology can be used in parallel with molecular methods for

sample preparation, or as a standalone capture and plating method. One area of microbiology that has benefited tremendously is the detection of verocytotoxin producing *Escherichia coli* (VTEC). The issue with this important group of zoonotic enteric pathogens is that they are not always biochemically distinct in comparison to non-toxigenic *E. coli* variants and are often present at low numbers in a high background of competitive microorganisms. In this instance, IMS is an excellent tool to effectively capture, concentrate and isolate the organism of interest (Wright *et al*., 1994). The standard method is simple and starts with the mixture of the test solution with specific IMS beads for the target microorganism. The test solution may be a homogenized matrix or a sample from secondary enrichment. After the beads have been mixed and bound to the specific target they can be immobilized onto a magnet. With the beads held in place, the excess supernatant can be removed, and the beads washed to remove unbound bacteria. This process is repeated until only the antibody bound organism remains. These beads can then be spread on an agar plate or utilised in another end-point test. When using beads to capture molecules, there is often a step to detach the analyte from the bead, however since the bead will not interfere with the organism's ability to replicate it is not necessary for microbiological purposes. The key property for the raw magnetic particle is that it has an unreactive surface so that the only interaction is with the bound antibody. Many bead types utilize polystyrene as a polymer coating, but other materials are available that have different properties, such as zirconium which is a highly resistant metal. The bead core is often made from iron oxide due to the superparamagnetic properties but must be coated to prevent iron toxicity when interacting with cells. An important property when it comes to performance of an IMS bead is the immobilization of the antibody on the beads surface. There are many methods/chemistries that can be employed including covalent coupling, adsorption and affinity binding. Not only does the coating technique have a huge effect on binding efficiency of the antibody, but also on how the bead will interact with nonspecific targets. The capture efficiency and specificity of IMS beads is largely due to the antibody bound to the magnetic bead. Difficulty arises for *Salmonella* when creating a species wide bead, as a cell surface antigen must be identified that is conserved and expressed by all the serological variants,

which number in the thousands. There are commercially available IMS products that target antigens of *Salmonella* spp. that have been shown to be superior to the standard ISO method (Cudjoe *et al*., 1994). By using IMS it is possible to effectively remove background microorganisms by specific capture and washing of the target organism. However, the specificity of the antibody is sometimes difficult to achieve due to the similarity of other *Enterobacteriaceae* that express very similar (if not identical) target antigen. It is possible to raise an antibody for a specific *Salmonella* serovar if a unique target can be found and antibody be raised against it.

1.6 Chromogenic media

Chromogenic media are formulations that contain compounds which are substrates for specific enzyme activity. When these substrates are hydrolysed by enzymes, produced by microorganisms, they release colourful insoluble dyes that build up inside the cells resulting in coloured colonies on the agar surface. Chromogens consist of two parts, a chromophore and a substrate. The most exploited chromogenic compounds are indoxyl derivatives pioneered by Ley in the late 1980s (Ley *et al*., 1988). When indoxyl is released it undergoes spontaneous dimerization in the presence of oxygen. This results in a visible colour appearance which depends on the halogenation of the molecule to which colour is presented. The substrate is the molecule to which enzyme activity will be targeted. The most utilised substrates in commercially available culture media are the glycosidases, including galactosidases, glucuronidases and glucosidases. There are a large variety of other substrates that are available ranging from phospholipases, sulphatases, esterases and amino peptidases. Indoxyl substrates are widely used due to their stability and low toxicity. However, there are many other alternatives, such as esculetin derivative 3-4-cyclohexenoesculetin (CHE) which forms a black chelate in the presence of iron (James *et al*., 1997). The first chromogenic media for *Salmonella* was described by Rambach in the early 1990s (Rambach, 1990). Rambach agar utilised the ability of *Salmonella* spp. to ferment propylene glycol which is visualised by the formation of red colonies. The chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal) is used to differentiate lactose

fermenting organisms by the formation of blue colonies. However, this agar was flawed, as it is unable to detect *S.* Typhi and β-galactosidase producing *Salmonella* (Kühn *et al*., 1994, Pignato *et al*., 1995). Later a chromogenic agar named ABC medium was developed by Perry *et al.* and used a dual chromogenic system with a modified DCA base (Perry *et al*., 1999). The two chromogenic substrates are 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-gal) and 3-4-cyclohexenoesculetin-β-D-galactoside (CHE-gal). These allow the agar to differentiate *Salmonella* from other *Enterobacteriaceae* by their ability to produce α-galactosidase in the absence of β-galactosidase. This results in blue/green *Salmonella* and black non-target organism. Other organisms than *Salmonella* produce α-galactosidase but this activity is masked by the CHE substrate. This masking reaction works well since the use of the CHE substrate results in a black colour that cannot be misinterpreted for the blue/green *Salmonella*. It would not be possible to use another indoxyl chromogen, such as Salmon-gal as the masking chromogen as the colour mix produced would be too subjective for an accurate diagnostic medium.

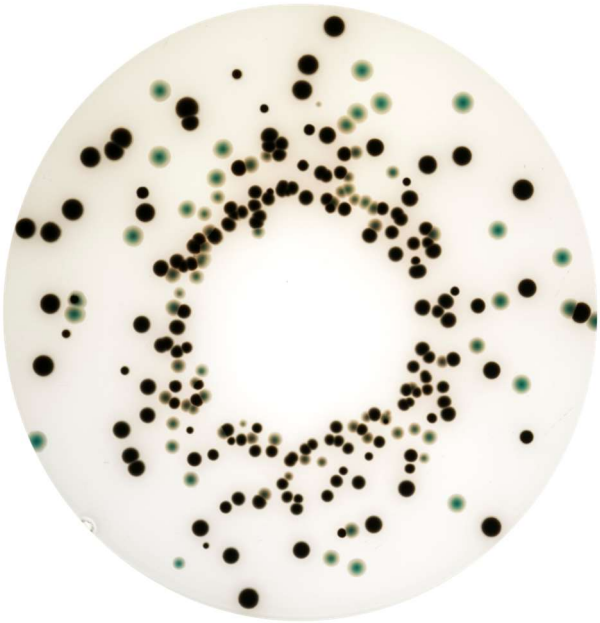


Figure 1.6 The appearance of *Salmonella* (green colonies) and *Enterobacter* (black colonies) on ABC agar (Lab M).

ABC has been shown to offer an improvement over traditional media for *Salmonella* isolation and detection (O'Neill *et al*., 2003), but suffers from the issue of its inability to detect lactose positive *Salmonella* and furthermore, some *Salmonella* do not express α-galactosidase on this medium. ABC is unable to detect lactose positive *Salmonella* because lactose is a substrate of β-galactosidase meaning lactose positive *Salmonella* will appear black on the agar surface. The biggest issue with the earlier generations of *Salmonella* chromogenic agars was the fact that not all *Salmonella* species utilised the target substrate, so inherently the formulations would fail to positively identify some isolates. A new substrate was described for *Salmonella* based on its ability to produce esterase against a C_8 ester known as caprylate (Pontello *et al*., 1987). This is conjugated to the fluorophore 4-methylumbelliferyl and the compound was called MUCAP. It was not stable enough to be incorporated into media but was instead dissolved in hexane and dropped on suspect colonies. This would lyse the cells and if present, the enzyme would hydrolyse the compound resulting in blue fluorescence under a UV light source. The compound proved to be a sensitive confirmatory test for many diagnostic agars, but lacked specificity due to relatively high false positive rates (Humbert *et al*., 1989). Also, due to the

destructive nature of the test, subculture had to be performed to preserve the isolate prior to testing. Subsequently, further versions of the substrate were made available through improved knowledge of syntheses resulting in new chromogenic agars. The first utilised 4-[2-(4-octanoyloxy-3,5-dimethoxyphenyl)-vinyl]-quinolinium-1-(propan-3-yl carboxylic acid) bromide (SLPA-octanoate; bromide form) and was called Chromogenic Salmonella Esterase (CSE) agar (Cooke *et al*., 1999). This presented with burgundy *Salmonella* colonies with other organisms appearing colourless or yellow to cream. The agar performed well in the initial study, but due to the high level of lactose in the formulation would not perform well with lactose positive *Salmonella.* The most successful esterase based agar for *Salmonella* was developed by CHROMagar Microbiology in Paris (Gaillot *et al*., 1999). This was the first commercial agar plate to utilise 5-bromo-6-chloro-3-indoxyl-caprylate which results in magenta colonies when hydrolysed. The ability of esterase to act upon the caprylate substrate is not specific to only *Salmonella* but many other organisms. Many other *Enterobacteriaceae,* such as *Enterobacter* and other organisms like *Pseudomonas* species and the yeast *Candida* can utilise the substrate. However, media utilising the caprylate substrate either employ a masking chromogen in the case of organisms like *Enterobacter* or a selective system to inhibit organisms like *Pseudomonas* and *Candida*. The first iteration of the CHROMagar product used X-gal as a blue mask for *E. coli* and coliforms and sodium deoxycholate and cefsulodin to inhibit *Candida* and *Pseudomonas,* respectively. Using β-galactosidase activity to mask the production of esterase of non-*Salmonella* spp. works well, but this also prevents the correct identification of β-galactosidase positive *Salmonella.* This product was later redeveloped to substitute X-gal for 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside (X-glc). This chromogen combined with an appropriate selective system allows for masking of non-*Salmonella* esterase activity without preventing the detection of lactose positive *Salmonella*. Esterase activity is much more conserved amongst all *Salmonella* species (Carinato *et al*., 1998, Goullet, 1977) than other enzymes utilised previously in *Salmonella* media, making it a more sensitive target. As such, there are many commercial products that use chromogenic caprylate substrates paired with X-glc and a

selective base. Examples include Brilliance Salmonella Agar (Oxoid), RAPID' *Salmonella* Medium (BIO-RAD), ASAP (bioMérieux) and *Salmonella* Chromogenic Agar (Conda).

Figure 1.7 *Salmonella* growing on *Salmonella* Chromogenic Agar (Conda), utilising the magenta caprylate chromogen resulting in pink/purple colonies.

All the current generation of chromogenic agars for *Salmonella* utilising the caprylate substrate have an inherent flaw. There are some *Salmonella,* such as *S.* Dublin that produce very little activity against the substrate and result in colourless colonies after 24 hours incubation (Gray *et al.*, 2003).

1.7 Culture media formulation

Culture media, as described in this chapter, is an integral part of currently used *Salmonella* detection methods. Culture media formulations contain various components that are required for optimal growth and recovery of the target microorganisms.

1.7.1 Growth promotion

Technically it would be possible to create a completely defined media from refined chemicals, taking the optimal amounts of each required amino acid and minor components. However, not only would this be extremely expensive but complex to formulate and impractical to manufacture at scale. Peptones are water soluble, protein digests of animal and plant matter, which are available from various sources as dehydrated powder. They contain the carbon and nitrogen sources required for bacterial growth. Since there is a wide array of peptone sources that are suitable it is easier to select appropriate variants or mix and/or supplement to achieve the performance required. The peptone base is the common starting point for any culture media development. This is because a given medium needs to meet the nutritional requirements of the desired target organism/s. Since peptones are often the major components in a formulation they are key issue for compatibility, with the other components of the medium. Materials can be incompatible due to performance issues or for physical problems like clarity and precipitation. Depending on their source peptones can have greatly different effects. This can be due to the amino acid ratio, peptide chain length, available metal ions and pH in solution or presence of other materials that may affect growth.

Peptones provide an amino acid or nitrogen source required to support growth in culture media. However, they tend to be lower in other essential micronutrients due to the nature of manufacture and source material. This means that a medium only containing peptones as a source of nutrients may take longer to yield the expected colony size or diagnostic feature of a medium. The most common other culture media supplement is yeast extract. Yeast extract is the soluble extract of autolysed yeast cells which can provide a source of vitamins, micronutrients and growth factors. It can also contain relatively large amounts of carbohydrates such as mannose. An alternative is beef extract and is typically made from low fat meat sources and contains peptides, organic acids, vitamins and minerals.

Carbohydrates are also normally a key component in many culture media formulations. These repeating sugar units provide a readily usable carbon source that allows for increased growth rates

and biomass. In culture media for *Salmonella* lactose and sucrose are often used as a method for differentiation against other *Enterobacteriaceae*, since most *Salmonella* cannot ferment them and subsequently produce acid and/or gas.

1.7.2 Selective agents

Since a sample is likely to contain multiple different microorganisms as well as a potential pathogen, it is necessary to employ selective pressure in favour the growth of the target organism. Bile salts No. 3 is a mix of the two major bile fractions used in culture media; sodium deoxycholate and sodium cholate. The mixture is typically in a ratio of 55:45 in the favour of deoxycholate. Sodium deoxycholate major purpose is to inhibit non- bile tolerant bacterial including a wide range of Gram positive organisms. Sodium cholate generally improves growth of bile tolerant organisms whilst offering a level of selectivity in a similar manner to sodium deoxycholate. These two components are purified individual components of crude ox bile. Trisodium citrate is a chelating agent and can bind out of solution divalent cations such as magnesium and calcium ions. *Salmonella* are more tolerant of trisodium citrate than other enteric organisms and can strip the bound cations as well as use it as a carbon source. Both bile acids and citrate are active against bacterial cell membranes causing osmotic stress, permeability and leakage, interfere with protein folding / cause protein dissociation and alter enzyme activity (Begley *et al*., 2005, Lee *et al*., 2001).

1.7.3 Diagnostic features

Besides published typical morphologies, microbiologists rely on diagnostic culture to differentiate microorganisms. To differentiate or help alert for the presence of a given microorganisms, compounds can be included to give a visual signal based on various reactions. One of the most long standing is the use of pH indicator dyes (Atlas, 2005), which change colour to correspond with the pH of the medium. These allow for the visualisation of carbohydrate fermentation or protein deamination by the detection of the subsequent acid or alkali, which changes the pH of the media. Other biochemical colour change markers are used such as the reduction of compounds that yield to colour change

reactions, like the hydrogen sulphide reaction in XLD. Chromogenic and fluorogenic substrates are the most recent diagnostic compounds to be employed in culture media.

1.7.4 Buffers

The correct pH of a growth medium is crucial for optimal growth of a given microorganism. During growth of a microorganism by-products may be produced that shift the pH of a medium. Incorporation of buffers allows for this change to be resisted and the pH held at an optimum value. There are various types of buffering agents employed in culture media, but phosphates are the most common due to low cost and high availability (Corry *et al*., 2011).

1.7.5 Gelling agents

Solid media requires a gelling agent to form a physical surface for microorganisms to grow on. First discovered by Walther Hesse in the 19th century (Russell and Cohn, 2012), agar (from agarphyte seaweed) is the standard gelling agent used in microbiology. Previous researches had found that other agents such as gelatine were unsuitable, since it would melt at high temperatures and would be digested by bacteria. Angelina Hess (the wife of Walther Hesse), gave her husband the idea as she used it to make jellies that would not melt in warm conditions. As well as providing structure, agar also contributes to the availability of metal cations and can influence multiple diagnostic reactions.

1.8 Aims

The aims of this study were to:

- Develop an improved diagnostic chromogenic agar for *Salmonella*.
- Shorten the time to result of *Salmonella* testing by reducing the enrichment time.
- Pair the new diagnostic agar with the new rapid enrichment protocol and carry out a study on retail produce.
- Investigate the use of IMS technology for the improved recovery of *Salmonella*.
- Investigate possibility of improving methods for *S.* Typhi detection.

The first three aims are primarily concerned with addressing the problem of foodborne *Salmonella* contamination in Europe, by improving the detection methodologies. The final two aims are concerned with addressing the problem of *S.* Typhi and resulting typhoid fever in endemic areas such as sub-Saharan Africa. By splitting the focus towards two very different burdens of *Salmonella*, this study hopes to address the pathogen from a global perspective.

Chapter Two

General Methods

2.1 Raw material handling

Bulk raw materials required to produce all culture media formulations were supplied by Lab M Ltd (Bury, UK). To produce a given formulation the required materials were weighed out at the correct g/l for the volume of media being prepared. The materials that require milling to be soluble were dissolved in an appropriate solvent such as *N*,*N*-Dimethylformamide (DMF), to make a solution that could be added to reconstituted media. Compounds that were added at levels that were difficult to accurately weigh (<0.02 g/l), were dissolved in water (or appropriate diluents) and added as a solution to reconstituted media. Commercial media was prepared to manufacturer's instructions and was supplied by Lab M Ltd, unless stated otherwise.

2.2 Water

All water used for preparation of culture media and all other reagents was either deionised or reverse osmosis (RO) purified water.

2.3 Preparation and sterilisation

Once prepared media was rehydrated and mixed thoroughly to ensure complete dissolution. Then any required solutions were added volumetrically before sterilisation. The chromogenic agar formulations described in Chapter 3 were sterilised by bringing to the boil. This was typically done in 500 ml volumes in Erlenmeyer flasks, covered with tinfoil and heated on a halogen hob (as shown in Figure 2.1).

Figure 2.1 The typical appearance of a preparation being sterilised by boiling on a ceramic hob.

The total volume of the flask was at least double the volume being prepared, so that adequate space was available for boiling to occur without spillage. During heating media was frequently agitated, to ensure proper mixing and to prevent localised heating. Once media was brought to the boil it was removed from the heat source and cooled to 47-50 °C, in a water bath before pouring into Petri dishes. All other media was sterilised according to manufactures instructions, typically by processing in an autoclave in Duran bottles for 15 minutes at 121 °C.

2.4 Physical characteristics testing

The major parameter examined during the production of culture media was the pH. This was tested using a calibrated laboratory pH meter (PHM220, Radiometer Analytical SAS). All media was tested after sterilisation once the media had reached 25 °C. This was done by storing dispensed media in a 25 °C incubator for at least 2 hours.

2.5 Incubation

After inoculation media was incubated using various precision cooled incubators (LEEC Ltd). Media was never pre-warmed to the target temperature, but was equilibrated to room temperature before inoculation and incubation.

2.6 Bacteriological testing

2.6.1 Culture preparation and dilution

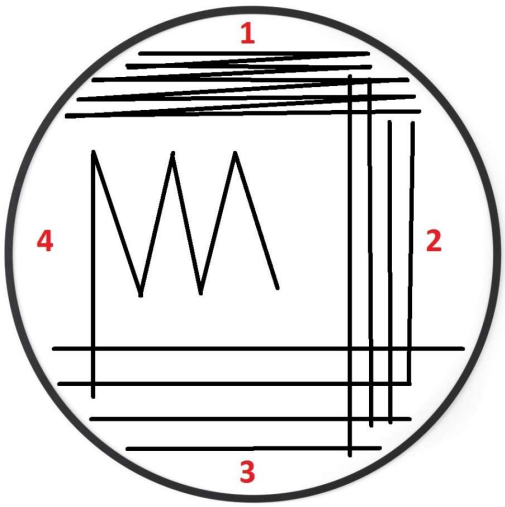
Cultures were revived from storage at -80 °C by streaking a cryopreservation bead (Protect, TSC) onto a tryptone soy plate (TSA) and incubating at 37 °C for 18-24 hours. The resulting growth was checked for purity (identical morphology) and subcultured into tryptone soy broth (TSB), by picking three identical colonies with a sterile loop. The broth was then incubated at 37 °C for 18-24 hours. The required cell density inoculum was prepared by serial dilution in maximum recovery diluent (MRD). This was typically done with 4.5 ml MRD in glass bijoux, sequentially adding 0.5 ml of culture suspension to create a 1 in 10 dilution. Culture suspensions in MRD were then kept for a maximum of two hours at ambient temperature before use.

2.6.2 Quantitative recovery testing (solid media)

Productivity testing, or the ability to recover organisms on an agar medium was attained by quantitative recovery testing. Culture suspensions as described in 2.6.1 were inoculated onto the agar surface either by spreading 50 µl with a sterile L-shaped spreader, or by using an automated spiral plater. The Whitley automated spiral plater (WASP, Don Whitely Scientific, Shipley UK) was used to evenly distribute organisms on an agar surface, by depositing 50 µl of a dilution in a circular pattern. To attain the productivity of a test formulation, plating was done in parallel with TSA as a control. The number of colony forming units (CFU) was counted on the control plate and compared to the test formulation. Productivity was then calculated as a percentage of colonies recovered, compared to the control.

2.6.3 Qualitative recovery testing (solid media)

Ability to support growth and/or production of typical morphology on agar was attained by qualitative recovery testing. This was done by using a 5 µl streak of a culture dilution, to fully streak across the agar surface in a pattern as presented in Figure 2.2. The resulting growth was then scored by observing where along the streak the organism had grown to. Plates that had growth at the primary inoculum only were scored with a single +. Plates that had growth up to the secondary inoculum were scored as ++. Finally plates that had growth to tertiary growth and beyond were scored with +++. The final streak on the pattern in Figure 2.2 (position 4) was typically used to observe isolated single colonies. This allowed for observation of colony colour, shape and size.



NG	No growth
+	Light growth (growth at primary inoculum only)
++	Medium growth (growth to secondary inoculum only
+++	Heavy growth (growth to tertiary inoculum and beyond)

Figure 2.2 Streaking pattern used for a full plate streak, starting at position 1 and progressing to position 4.

2.6.4 Qualitative recovery testing (liquid media)

The ability to support growth in liquid media (broth) was typically performed by inoculating 50 µl of a culture dilution into 10 ml of broth. After incubation growth was scored from + to +++ by comparing the intensity of growth (turbidity) by eye.

2.6.5 Latex agglutination testing

To confirm presumptive positive *Salmonella* colonies on agar, a commercial latex agglutination kit was used. *Salmonella* test kit (DR1108) was sourced from Oxoid Ltd (Basingstoke UK) and allowed for the quick confirmation of presumptive identification. A suspect colony was emulsified in a drop of saline using a sterile loop, on an examination card. The suspension was then observed for any autoagglutination. If not, a drop of latex reagent was added, and the suspension was gently mixed by rocking the card. If clumping or agglutination was observed within two minutes of mixing, the isolate was confirmed as a positive *Salmonella*. Control tests were also carried out with each group of tests, with the supplied positive control solution and the negative control saline solution. This was to ensure the test kit had not been contaminated and that positive agglutination matched the appearance of the control.

2.7 Dehydrated culture media manufacture

To increase confidence in the results and allow for larger scale testing of novel formulations, small batches of dehydrated culture media (DCM) were manufactured after the initial development. This allowed for multiple tests to be carried out from the same batch of materials, ensuring the results were accurate and not due to batch to batch variations of natural raw materials (e.g. peptones). Blending of powders of a homogenous particle size (such as peptones and agars) was done in a barrel with >50 % headspace, using an inverting barrel mixer. Materials that had a different (larger) particle size were milled using an ultra-centrifugal mill (ZM 200, Retsch), with a mesh screen size of 0.5mm. This process was also done with any materials that were <3 % of the blend (such as antibiotics), to

improve homogeneity. The milling process created a uniform particle size of all materials, which then could be blended via barrel mixing.

Chapter Three

Chromogenic Agar for *Salmonella* Esterase

3.1 Introduction

Salmonella is one of the biggest causes of food-borne outbreaks in the European Union and in 2015 resulted in over 90,000 cases of hospitalisation due to salmonellosis (EFSA, 2016). Compared to other commonly reported zoonotic agents such as *Campylobacter,* we have had the ability to isolate the bacterium for many years. As such there are probably more culture media formulations described for *Salmonella* than any other specific pathogen. There are many selective and diagnostic media available for the detection of *Salmonella* based on a specific set of biochemical and physiological rules. These rules include the inability to ferment lactose and sucrose, decarboxylate lysine and produce hydrogen sulphide (H_2S) from thiosulphate and other sulphur sources. Xylose Lysine Deoxycholate (XLD) agar uses these parameters to differentiate *Salmonella* from other bile tolerant enteric organisms by their formation of black colonies on the agar surface. Due to the extensive diversity of bacterial species there is high incidence of atypical biochemical profiles that lead to misidentification on culture media (Lin *et al*., 2014).

Figure 3.1 A negative H_2S reaction of a *Salmonella* isolate on XLD agar resulting in pale colonies.

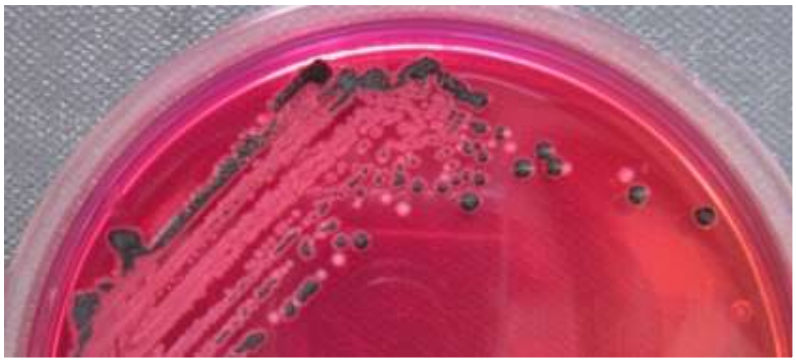


Figure 3.2 A positive H_2S reaction of a *Citrobacter* spp. isolate on XLD resulting in black colonies.

Figures 3.1. & 3.2 both show incorrect biochemical reactions on XLD that would result in either failure to identify *Salmonella* or which would require further confirmatory testing of a non-target organism. Chromogenic media offer an advantage over traditional media because of their ability to visualise enzymatic activity instead of a purely biochemical phenotype. Chromogenic substrates are incorporated into agar formulations and yield coloured colonies as a result of enzyme expression. Currently the most popular chromogenic agars visualise esterase activity of *Salmonella enterica* serovars by the cleavage of the chromogenic substrate, 5-bromo-6-chloro-3-indolyl caprylate more commonly known as magenta-caprylate or octanoate (due to the 8-carbon chain). Esterase activity of non-*Salmonella* is either masked by a second chromogen, X-glucopyranoside or growth is inhibited by selective components in the agar. This results in pink/purple *Salmonella* and blue non-target *Enterobacteriaceae,* such as *Enterobacter* spp. and *Klebsiella* spp. Other non-target bacteria that can grow such as some *Shigella* spp. and *E. coli*, present either white or colourless colonies. The plating media for cultural *Salmonella* detection methods is crucial since it is the only diagnostic part of the method that can identify the pathogen. Failure to identify at the plating stage will result in incorrect reporting of the status of a given foodstuff.

All the current generation of chromogenic agars for *Salmonella* utilising the caprylate substrate have an inherent flaw. There are some *Salmonella*, such as *S.* Dublin that produce very little activity against the substrate and can result in colourless colonies after 24 hours incubation (Gray *et al*., 2003).

Figure 3.3 Conda Chromogenic *Salmonella* Agar (Left) and Oxoid Brilliance *Salmonella* Agar both exhibiting weak to no chromogenic reaction with *S.* Dublin.

Due to the 8-carbon chain substrate, agars that use magenta caprylate are often referred to as C8 esterase agars. *S.* Dublin has very weak activity against the caprylate substrate as shown in Figure 3.3. In addition, due to the selective systems of several of the commercially available formulations, some *Salmonella* such as isolates of *S.* Gallinarum fail to grow within the 24-hour incubation time (See Figure 3.4). In both instances, such media result in false negative results that could result in the release of contaminated foodstuffs. Both *S.* Dublin and *S.* Gallinarum are host adapted serovars. *S.* Dublin is a significant pathogen that can lead to serious invasive gastroenteritis, and is one of the most isolated serovars from cattle in Europe (EFSA, 2016). *S.* Gallinarum is the causative agent of fowl typhoid, a severe disease of chickens and other galliforme birds (Gast, 1997). These serovars pose a significant threat to the food industry by causing disease and reducing yields, as well as a potential zoonotic threat.

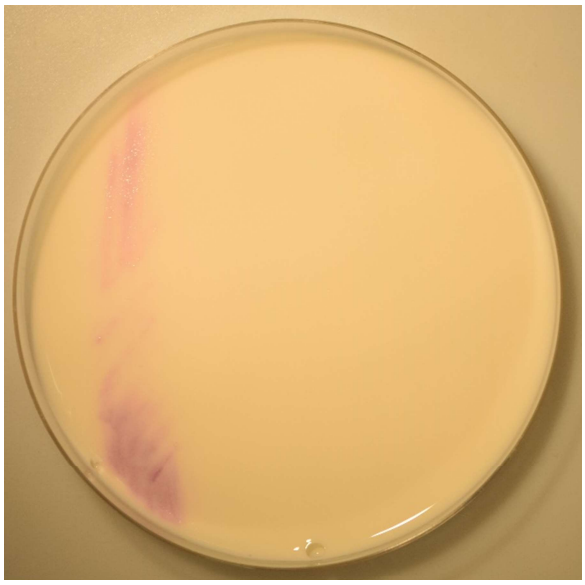


Figure 3.4 A 5 µl streak of a *S.* Gallinarum isolate (approximately >10^6 CFU) yielding a poor chromogenic reaction and growth response on Oxoid Brilliance *Salmonella* agar.

Some commercially available formulations can also result in a relatively high number of false positive results, due to non-target organisms presenting the same colour colonies as *Salmonella* on the agar. This, whilst less serious than a false negative result, consumes time and resource to resolve. The intended benefit of chromogenic agars over traditional formulations is that they have greater sensitivity, specificity and rely less on interpretation by the reader.

There is a clear need for an improved diagnostic agar formulation that can detect weak esterase producing and slower growing *Salmonella* species.

Magenta-caprylate, an indoxyl fatty acid ester chromogen is appealing for use in culture media for several reasons. Due to its relatively high use, it is cheaper than other less used substrates mainly due to the scale of production and availability through multiple vendors. Also, esterase appears to be relatively well expressed amongst most *Salmonella* compared to alternative substrates like X-α-gal, which can result in several false negatives on ABC agar (Personal Observations). If this substrate is to be used however, the previously mentioned flaws would have to be addressed to improve on currently available formulations. Commercially available agar that use magenta-caprylate do not state their

exact formulations, but instead are published with vague descriptions to protect the intellectual property of the owner.

Finally, from a practical perspective the new agar formulation should be easily prepared to lend itself to widespread use. Autoclave sterilisation is not possible in lower income countries that may lack facilities to do so. Also, many commercial products for this purpose require post sterilisation supplementation. This is unfavourable as it adds further complexity and cost. The formulation should also have at least a six weeks shelf life once prepared to reduce wastage of media.

The design goals for the new formulation were as follows:

- Use esterase activity as a marker for *Salmonella*
- Use a substrate for glucosidase activity to mask non-target organisms
- Improve the detection of weak esterase *Salmonella* like *S.* Dublin
- Improve the recovery of slow growing, sensitive *Salmonella* like *S.* Gallinarum
- Be able to detect atypical *Salmonella* commonly missed by traditional formulations such as lactose positive, H_2S negative and non-motile *Salmonella*.
- Have an incubation time of 18-24 hours with a temperature of 37 °C ± 1 °C
- The media should be prepared by boiling to sterilise
- To be a fully blended formulation with no supplementation required
- Have at least a one-year shelf life dehydrated and six weeks prepared

3.2 Materials and methods

All culture media materials such as peptones, agars and other chemical raw materials were obtained from Lab M Ltd, United Kingdom. All raw materials, unless otherwise stated were supplied by Lab M Ltd. Chromogens were sourced from Inalco S.p.A, Italy, Glycosynth, UK and Biosynth AG, Switzerland. All standard dehydrated culture media (DCM) was supplied by Lab M Ltd, and proprietary *Salmonella*

chromogenic agar was obtained from Oxoid Ltd, UK and Laboratorios CONDA, Spain. All dehydrated culture media (DCM) was prepared according to manufactures instructions.

Type cultures and wild type isolates were supplied by the QC & R&D departments at Lab M, as well as isolates provided by Dr Lizeth Lacharme-Lora at University of Liverpool, Leahurst Campus.

Table 3.1 *Salmonella enterica* species obtained from the University of Liverpool

Short Code	Designation	Serovar	Source (UK unless stated)
L1	501249 DT193 4,[5],12:i:-	Typhimurium-like	Pig
L2	*S.* Enteritidis 01-00493-2	Enteritidis	Human (USA)
L3	500360 U288 4,[5],12:i:-	Typhimurium-like	Pig
L4	SARB18 FB *S.* Enteritidis	Enteritidis	Human (USA)
L5	000398ST DT193	Typhimurium	Pig
L6	500343 STU288	Typhimurium	N/A
L7	ST F98	Typhimurium	Chicken
L8	ST D23580	Typhimurium	Human (Malawi)
L9	*S.* Gallinarum 287/91	Gallinarum	Chicken (Brazil)
L10	*S.* Typhimurium DT193	Typhimurium	Pig
L11	*S.*E P1251D9	Enteritidis	Chicken
L12	ST 244	Typhimurium	Greenfinch
L13	ST04327 DT194 4,[5],12:i:-	Typhimurium-like	Pig
L14	ST 4/74	Typhimurium	Cattle
L15	*S.* Arizonae (ONPG positive)	Arizonae	N/A

Table 3.2 Type culture collection organisms used. NCTC – National Collection of Type Cultures (UK), ATCC – American Type Culture Collection (USA), NCIMB – National Collection of Industrial Food and Marine Bacteria (UK).

Salmonella enterica* subsp. *enterica	**Designation**	**Short Code**
serovar Abony	NCTC 6017	Sala17
serovar Arizonae	NCTC 7355	Salar55
serovar Enteritidis	ATCC 13076	Sale76
serovar Poona	NCTC 4840	Salpo40
serovar Typhimurium	ATCC 14028	Salt28
serovar Virchow	NCIMB 50077	Salv77
serovar Dublin	ATCC 39184	Sald74
Non-Target Organisms	**Designation**	**Short Code**
Citrobacter freundii	NCTC 9750	Citf50
Citrobacter freundii	ATCC 43864	Citf64
Cronrobacter sakazakii	NCIMB 5920	Cs20
Enterobacter aerogenes	ATCC 13048	Entbaco5
Enterobacter cloacae	NCTC 10005	Entbaco5
Enterococcus faecalis	ATCC 29212	Entcfs12
Escherichia coli	ATCC 25922	Ec22
Escherichia coli	ATCC 8739	Ec39
Proteus mirabilis	ATCC 15290	Prom90
Proteus mirabilis	NCTC 11938	Prom38
Proteus mirabilis	NCIMB 13283	Prom83
Pseudomonas aeruginosa	ATCC 27853	Psa53
Pseudomonas aeruginosa	ATCC 9027	Psa27
Staphylococcus aureus subsp. *aureus*	ATCC 6538	Sa23
Shigella sonnei	ATCC 29930	Shso30
Shigella sonnei	NCTC 8574	Shso74
Shigella flexneri	ATCC 12022	Shfl22

All cultures were maintained at -80 °C on Protect cryopreservation beads from Technical Service Consultants Ltd, UK. Cultures were revived by plating a bead onto a non-selective Tryptone Soy Agar (TSA) plate and incubating at 37 °C for 18-24 hours. Culture suspensions were then produced by subculture into Tryptone Soy Broth (TSB) and incubating at 37 °C for 18-24 hours. Serial dilutions were prepared by dilution in Maximum Recovery Diluent (MRD) to achieve a target CFU/ml.

Agar inoculation was performed either by streaking 5 µl culture suspensions using sterile loops, or by 50 µl spiral plating using the Whitley Automated Spiral Plater (WASP) from Don Whitley Scientific, UK. Plates were then incubated at 37 °C for 16 and 24 hours, with counts, colour and morphology observed at both time points.

The approach was to develop the formulation by screening nutritional and selective systems compared to a control base. Improvements of productivity were assessed by calculating quantitative recovery verses a non-selective base such as TSA. Improvements of enzyme expression were assessed by colour intensity at various time points judged by eye. A core panel of type strains were initially used to represent the organisms expected to be encountered followed by an extended panel of *Salmonella* and non-target organisms once a beta prototype had been established.

Raw materials (peptones, agars etc) were dissolved in at least 500 ml of deionised water in Erlenmeyer flasks, sterilised by bringing to the boil and tempered to 47 °C before pouring. Compounds that could not be reliably and accurately weighed at this scale, such as antibiotics, were made as concentrated stock solutions and added by pipette before sterilisation. Compounds that were not completely soluble without fine milling, such as chromogens, were dissolved in Dimethylformamide (DMF) and added as concentrated solutions before sterilisation.

Once a beta prototype had been established, formulations were tested at food testing laboratories and validation test sites alongside alternative formulations. This allowed for field testing before the formulation was used in further studies. Exposure to real wild type isolates is essential, to ensure a formulation for the detection of an organism as diverse as *Salmonella*, is functional.

3.3 Results

3.3.1 Chromogens

It was observed during previous experimentation with magenta-caprylate that some form of bile acid was crucial for good expression of esterase and the resulting strong colour reaction for *Salmonella,* in a 24-hour incubation period in a simple agar formulation (Unpublished Work). Using magenta-caprylate, X-glc and a modified deoxycholate citrate agar (DCA) base, various sources of bile acids were tested to determine the optimal concentration and constituents. Crude hog bile, hyodeoxychlolic acid, sodium deoxycholate, sodium cholate and various sources of bile salt No. 3 mixtures were all tested in the base formulation. Hog bile was inferior to Ox bile in both chromogenic colour intensity and colony size. Hyodeoxychlolic acid also showed no improvement over Ox bile. Sodium deoxycholate on its own was not enough to induce a strong reaction and sodium cholate on its own did not result in as intense colour, as the bile salt No. 3 mixture. After a series of titration experiments the optimum ox bile/bile salts No. 3 mix was achieved. This considered the selectivity afforded by the mixture in terms of suppression of *E. coli* and *Citrobacter,* as well as the effect on the chromogenic reaction of *Salmonella.*

The first issue encountered with magenta-caprylate was non-specific cleavage of the chromogen substrate. Around the individual colonies a purple halo was noted that was not present on commercially available caprylate plates (see Figure 3.5). This alone was not a huge issue as it made *Salmonella* easy to see. The risk was that on a plate with various colonies this halo could make a colourless colony appear purple. This could be picked and tested further and result in a false negative. The greater issue was with the non-target organisms. *Enterobacter* has esterase activity as well as glucoside activity. However, the esterase activity of the actual colony is effectively masked and the purple halo appeared to be due to nonspecific cleavage (see Figure 3.6). Chromogenic compounds from different sources can have variable properties depending on the exact conditions of synthesis and preparation. Other batches of the same chromogen as well as other sources were tested, but they

all showed this nonspecific halo effect. Then the level of chromogen was reduced from 0.2 g/l to 0.1 g/l in decreasing increments of 0.02 g/l. This did not fix the issue but just reduced the intensity of the colour reaction to a point where the colour of the target colonies was not strong enough to differentiate. Various agars were tested as previous experience had shown that the gelling agent can have a massive impact on chromogenic reactions. However, none prevented or reduced the nonspecific halo effect. Following that, each compound in the media was sequentially removed to see what effect if any it had on the non-specific cleavage of the chromogen. The removal of compounds from the formulation had either no effect or reduced or prevented the chromogenic reaction.

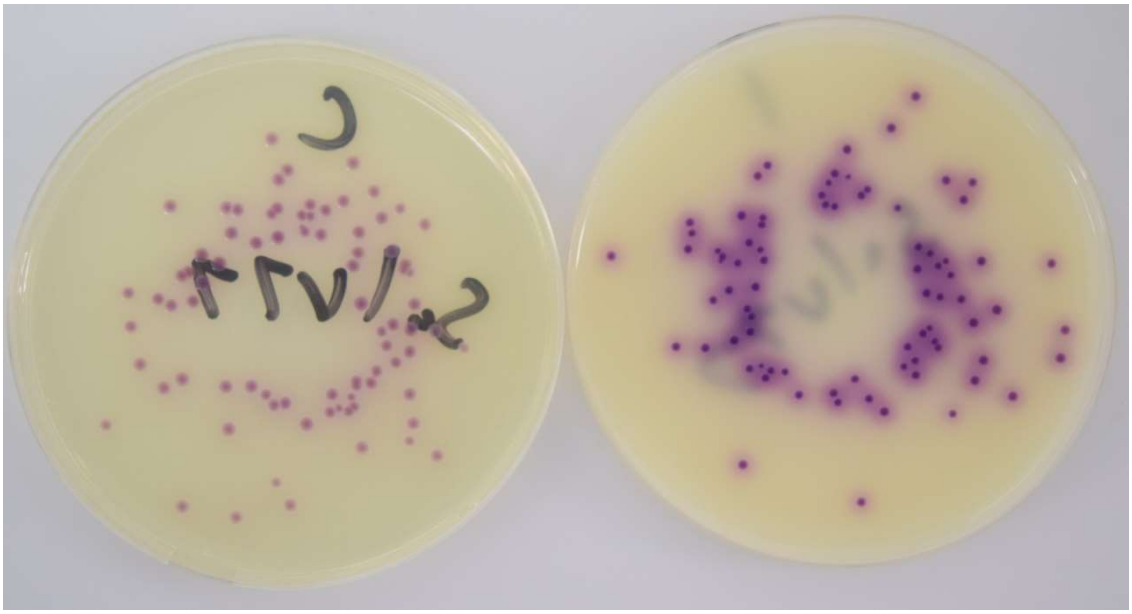


Figure 3.5 *Salmonella* Virchow NCTC 5742 inoculated onto Conda *Salmonella* Chromogenic Agar (left) and the caprylate based agar (right).

Figure 3.6 *Enterobacter aerogenes* ATCC 13048 inoculated onto Conda *Salmonella* Chromogenic Agar (left) and the caprylate based agar (right) after 24-hour incubation.

An experiment was also undertaken to determine the stability of the chromogen in the plate regarding pH, to test the hypothesis that pH shift due to metabolic activity was causing the issue. At this stage, no buffer was incorporated into the formulation. 100 µl of 0.2 M hydrochloric acid and 0.2 M sodium hydroxide was individually deposited onto the agar surface (see Figure 3.7).

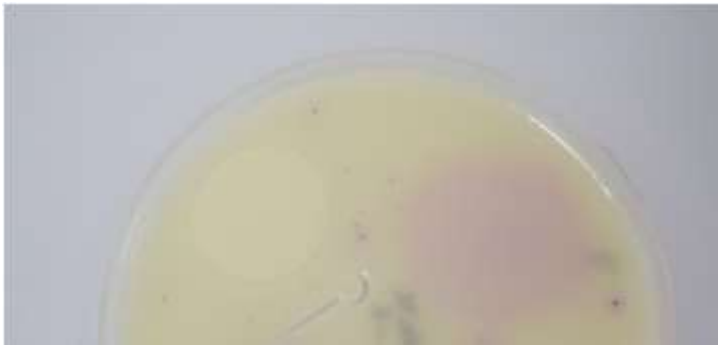


Figure 3.7 Drops of acid (left) and alkali (right) on the agar surface without the buffer present.

The acid caused a light white precipitate on the agar surface which was the bile salts precipitating, but no purple colour change was seen. The alkali caused a mild colour change around 5 minutes after it was placed on the agar surface. This was non-specific cleavage of the compound that could be related

to the halo effect, since as the organisms grow on the plate they will deanimate amino acids present in the peptone content causing a local rise in the pH. This also was an indication that the chromogen generally was not completely stable when incorporated into culture media. A phosphate buffer was incorporated into the medium to reduce this effect. This was established experimentally and from prior experience. The incorporation of a phosphate buffer did aid in the stability of the chromogen, but did not prevent the non-specific halo effect seen earlier on growing colonies. Since a reduction in compounds that promote the reaction also lessened the ability to identify *Salmonella* it was clear that something needed to be added to prevent the non-specific cleavage. Several compounds were screened with variable results. Copper sulphate dosed at a low level (<0.01 g/l) appeared to reduce the halo issue, but did not completely resolve it and reduced the average colony size of some of the slower growing *Salmonella,* such as *S.* Gallinarum. This was an issue as *S.* Gallinarum already produces small colonies without the copper sulphate. Potassium and sodium ferrocyanide salts were tested for their ability to prevent nonspecific esterase activity of the caprylate substrate. There was evidence in the literature that this may be a possibility as these compounds have been described in histological staining procedures to prevent extracellular esterase activity (Bancroft and Gamble, 2008). However, various concentrations failed to prevent the halo effect without majorly impacting the growth of *Salmonella*. Finally, a anionic surfactant sodium dodecyl sulphate (SDS) was tried as it is a common component in enteric culture media and it is well known as a denaturant of proteins (Bhuyan, 2010).

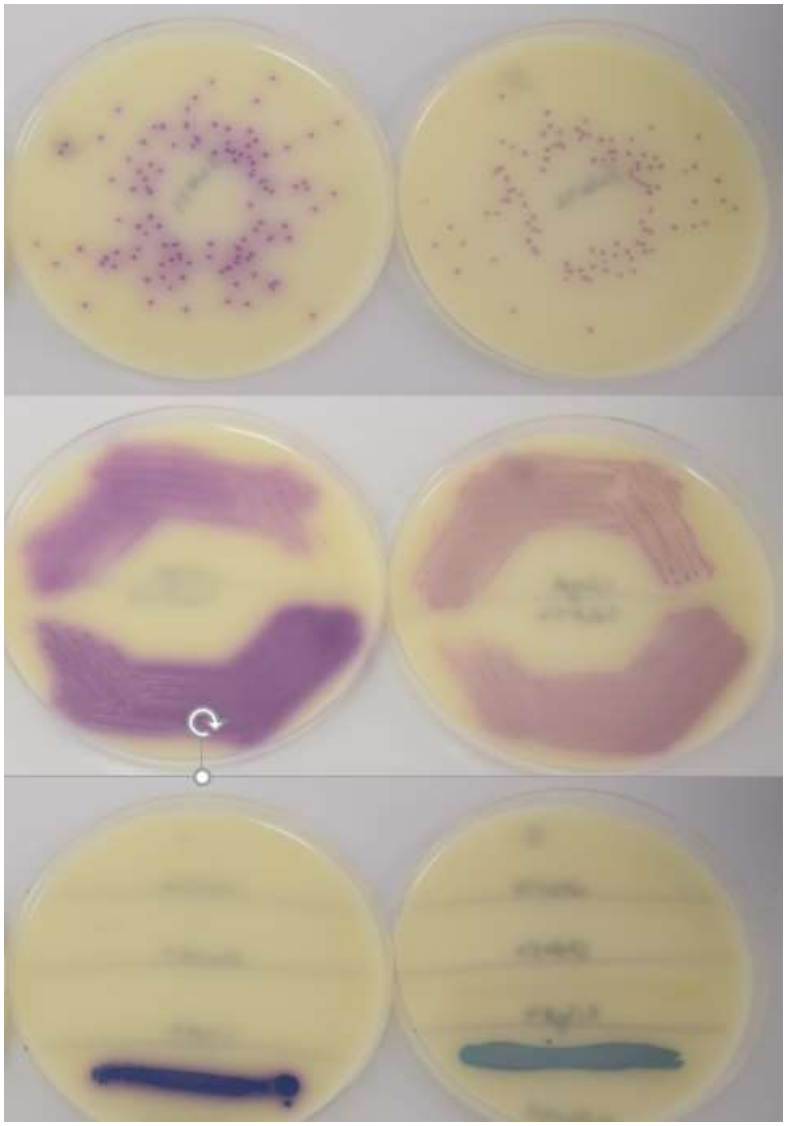


Figure 3.8 Bacterial test organisms growing on the chromogenic agar without (left) and with (right) the addition of 1 g/l SDS. Top plates are *S.* Typhimurium single colonies, middle plates are *S.* Gallinarum (top) and *S.* Enteritidis (bottom) half plate streaks and bottom plates show *Enterobacter aerogenes* growing from a line streak.

The addition of SDS at 1 g/l effectively stopped the halo effect in all organisms tested. It reduced the intensity of the magenta-caprylate colour but prevented the observed non-specific cleavage. Unfortunately, this addition reduced the selectivity of the media allowing organisms such as *E. coli* and *Shigella* spp. to dramatically increase in their qualitative recovery. This was not major issue as

such, since the organisms were colourless and thus could not be mistaken for *Salmonella.* They did however, cause more non-target crowding on the agar plate from mixed culture inoculations.

Parallel to this work, alternative esterase substrates were also tested. One of the more easily available compounds was 5-bromo-4-chloro-3-indoxyl nonanoate or X-nonanoate. This chromogenic substrate is like magenta-caprylate but is the blue indoxyl derivative and is a nine-carbon chain instead of eight.

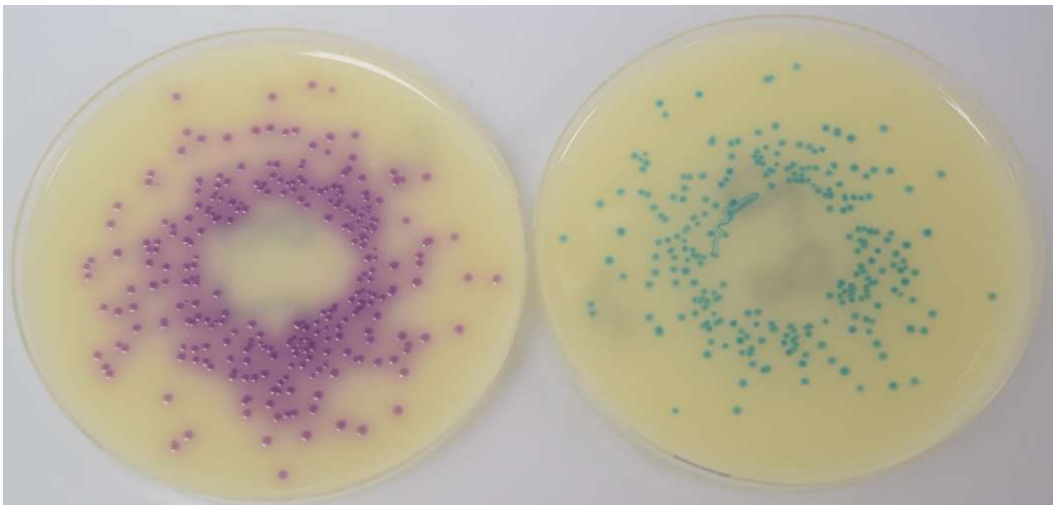


Figure 3.9 *S.* Typhimurium growing on the caprylate based plate (left) and the nonanoate based plate (right), both without the addition of SDS.

It was immediately clear that the nonanoate did not suffer from the same non-specific cleavage as the caprylate substrate. This was also evident when each compound was dissolved in DMF as the caprylate created a slightly pink solution, whilst the nonanoate was clear. The nine-carbon chain clearly was more chemically stable on its own and when incorporated into the agar base. The nonanoate also appeared to be superior to the caprylate regarding the weak esterase activity among serovars like *S.* Dublin giving a strong blue/green reaction compared to the caprylates very pale pink colour. X-nonanoate is commercially available and not protected by any patent relevant to this purpose so it is curious why others had not utilised it in culture media. However, the issue it does present how to properly mask non-target organisms that also have esterase. The magenta-caprylate uses the blue masking activity of X-glc for non-target esterase producing organisms like *Enterobacter* spp. This

system works because the colours are just different enough to differentiate when present in high numbers on the same plate. X-glc cannot be used with the X-nonanoate as they will essentially give the same blue/green colour making it too difficult to distinguish between the two. Other common chromophores do not offer strong enough differentiation, for example green, yellow or salmon would either be too close to the original colour to mask or be too weak/light to properly mask utilisation of the X-nonanoate. This is where the patent protected (James and Armstrong, 2001) 3,4-cyclohexenoesculetin (CHE) derivatives are highly useful due to their intense black colouration when present with iron. CHE-glc was incorporated into the base formulation at 0.2 g/l with 0.5 g/l ferric ammonium citrate as an iron source. This results in blue/green *Salmonella* and black CHE-glc reaction, effectively masking the esterase activity of non-target esterase producing organisms. Both caprylate and nonanoate formulations were taken forward for further testing even though the nonanoate appeared to be superior. This was in case there were a significant number of isolates that gave false reactions with nonanoate compared to the caprylate.

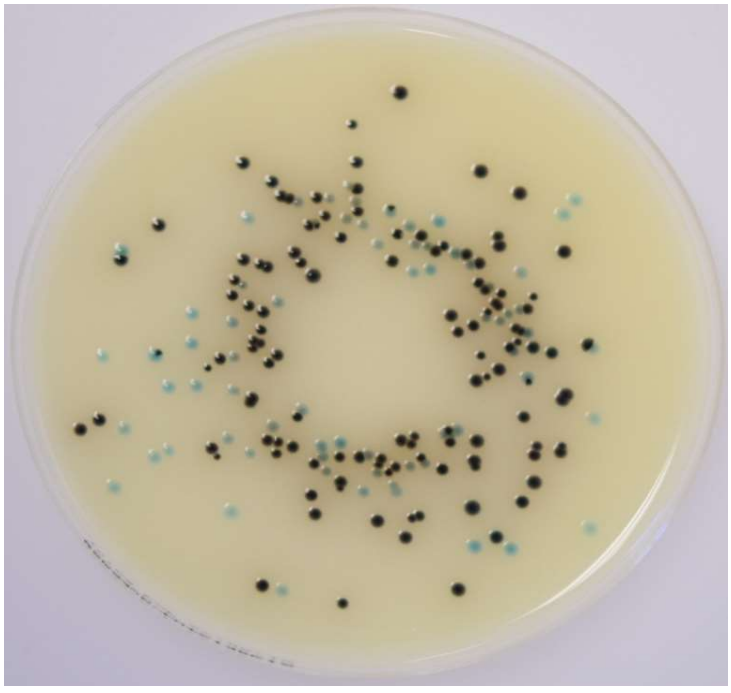


Figure 3.10 A mixed culture inoculation of *S.* Typhimurium (blue/green) and *E. aerogenes* (Black) on an X-nonanoate/CHE-glc formulation.

The formulations in Table 3.3 were prepared to test further with a wider range of organisms, matrices and protocols. The nonanoate base contains ferric ammonium citrate to allow functionality of the CHE masking chromogen. The caprylate base contains SDS to prevent the substrate from undergoing non-specific cleavage. SDS is traditionally used as a selective agent against Gram positive organisms. In this formulation SDS caused an increase in bile tolerance of the non-target organisms. This caused a reduction in the ability to supress organisms like *E. coli*. Thus, a further 2 g/l bile salts No. 3 was incorporated into the caprylate base, to reduce the impact on selectivity caused by the synergistic effect of SDS and bile salts. Both formulations are sterilised by boiling since highly selective properties of the base allow for this method of sterilisation and any increase in thermal exposure would be detrimental to the antibiotic content.

Table 3.3 Final formulations of both caprylate and nonanoate chromogenic *Salmonella* agar bases.

Nonanoate Base

Compound	g/L
Beef Extract	2
Pork Heart Infusion	1
Meat Peptone	5
Vitamin Mix	1
Sodium Pyruvate	0.5
Tri Sodium Citrate	8.5
Kaolin	7.5
Bile Salts No 3	2.5
Bacteriological Ox Bile	1
Disodium Hydrogen Phosphate	5.6
Potassium Dihydrogen Phosphate	1.4
Cellobiose	0.5
Ferric Ammonium Citrate	0.5
Novobiocin	0.015
Cefsulodin	0.005
Agar No 2	12.5
X - Nonanoate	0.17
CHE - Glucoside	0.2
	49.89

Caprylate Base

Compound	g/L
Beef Extract	2
Pork Heart Infusion	1
Meat Peptone	5
Vitamin Mix	1
Sodium Pyruvate	0.5
Tri Sodium Citrate	8.5
Kaolin	7.5
Bile Salts No 3	4.5
Bacteriological Ox Bile	1
SDS	1
Disodium Hydrogen Phosphate	5.6
Potassium Dihydrogen Phosphate	1.4
Cellobiose	0.5
Novobiocin	0.015
Cefsulodin	0.005
Agar No 2	12.5
Magenta Caprylate	0.17
X - Glucoside	0.2
	49.89

3.3.2 Peptones

The formulation contains a mix of bovine and porcine peptones supplemented with beef extract and a vitamin supplementation dispersed onto an ultra-filtered pancreatic digest of casein carrier. The meat peptone is of a bovine source (mainly bovine lung) and is an enzymatic digest. It has excellent growth promoting properties in liquid and solid media and is a very common peptone in culture media. Due to its rich nutritional properties, it is often employed in selective culture media for *Salmonella* since it allows them to overcome the selectivity employed, especially bile acids. It is particularly good at improving growth rates of many microorganisms compared to other peptone sources. This improves diagnostic media since if the organism is growing faster you will get a quicker diagnostic reaction, for example chromogenic or biochemical. The pork heart infusion is a peptone source similar to the meat peptone but is sourced from a pancreatic digest of porcine heart tissue. Peptone sources and combinations were screened by testing various combinations and concentrations in the same base formulation. This approach was slow as a large amount of experiments had to be carried out to properly screen for the optimal composition from all available materials. In this instance, mainly *Salmonella* cultures described in Table 3.2 were tested for productivity and time to a visible chromogenic colour change. Recovery counts were calculated by comparing to counts on non-selective TSA, and the chromogenic reaction was compared by eye between each tested formulation. As per many *Salmonella* agar formulations, enzymatic digest of meat gave the best reactions. Supplementation with pork heart infusion however, resulted in a more intense chromogenic reaction, giving a deeper colour reaction compared to the same amount of extra meat peptone. Other primary peptone sources were also explored such as both acid and pancreatic digests of casein, soya and gelatine peptone. The first observation was that a sole peptone source was inferior to a mix, yielding smaller less intensely coloured colonies. Acid hydrolysed casein yielded small colonies for some target organisms such as *S.* Gallinarum suggesting that this amino acid source is less favourable to this organism. It also could be because of a lack of tryptophan or a higher level of sodium chloride (NaCl), both of which are features of the production of this material (tryptophan is destroyed by the acid

hydrolysis and NaCl is a by-product of the neutralisation process). Soya peptone and tryptone produced acceptable growth but were inferior to meat peptone.

3.3.3 Extracts and vitamins

The beef extract further improved the nutritional properties of the peptones without impacting upon selectivity. The vitamin mix stated in the formulation is a mixture of thiamine (vitamin B1), pyridoxine hydrochloride (vitamin B6), nicotinic acid (vitamin B3), pantothenic acid (vitamin B5) and folic acid (vitamin B9) blended onto an ultra-filtered casein peptone carrier. An ultra-filtered casein peptone was used because of its low variability (compared to other peptones) and low antagonistic properties because it has been ultra-filtered. The carrier is required to effectively disperse the vitamins, which are in very small quantities into a powdered blend. Sodium pyruvate is added as a growth enhancer with a primary function to improve the recovery of stressed and/or sub lethally damaged cells. Sodium pyruvate is an intermediate in the glycolysis pathway and can be used as a carbon source. It also is a scavenger of reactive oxygen species such as peroxides, these can cause stressed cells to either fail to grow, or to grow more slowly. Even though its use in culture media is widespread and well known, an experiment was carried out to demonstrate its effectiveness. *S*. Typhimurium ATCC 14028 was grown for 18 hours in tryptone soy broth (LAB004, Lab M) at 37 °C. The culture was then diluted down in maximum recovery diluent (LAB103, Lab M), so that 100 µl of the suspension contained ~80-120 CFU. 1 ml of this dilution was heated at 55 °C for 3 minutes. A portion of the same dilution was untreated and plated on the agar base (with and without sodium pyruvate) to determine the expected number of recovered cells. The heat-treated portion was also plated onto the agar base with and without sodium pyruvate. All plates were incubated at 37 °C for 24 hours and colonies exhibiting typical morphology were counted. This experiment was repeated three times and, in all cases, a higher number of heat stressed cells were recovered on the base containing pyruvate. As expected, sodium pyruvate improves the ability to recover heat stressed cells.

3.3.4 Opacity agent

Kaolin is hydrated aluminium silicate derived from aluminium oxide and has a variety of medical and industrial uses. In the formulation, it is used to make the agar opaque giving it a matt white background. This is done for two reasons, firstly to make counting easier as coloured colonies will appear on a white surface. This means they can be seen earlier and clearer. Secondly the kaolin masks any potential precipitation issues that may be experienced. Varying levels of precipitation can be seen in media that contain bile acids, citrate and phosphates. Due to the complex nature of the formulation, relatively high concentrations of several components and the nature of bile acids to precipitate protein mean that it is possible to get what looks like aggregated crystals forming in the media on storage. Mostly this precipitation is purely an aesthetic problem, and performance is maintained. Kaolin masks any underlying aesthetic problems which otherwise may make the formulation less commercially pleasing. During development, it was noticed that when prepared in a larger bulk (>4 L) the formulation sometimes resulted in a grainy white appearance instead of a smooth opaque finish. After several experiments with sterilisation conditions, it was discovered that to achieve a matt finish of the kaolin, it was critical that the powder was completely dissolved prior to heating and that the media reached at least 100 °C.

3.3.5 Buffer components

The disodium hydrogen phosphate and potassium dihydrogen phosphate create a buffered system when incorporated into the formulation, that results in a pH range of 7.2 ± 0.2 at 25 °C depending on the other raw material properties. A buffer was originally added to help prevent non-specific cleavage of the chromogenic compounds and generally aid in the stability of the media. These specific phosphate compounds were selected, because previous experience had shown that when combined with bile acid selectivity, the result was a selective system that was harsher towards enteric organisms other than *Salmonella*. The buffer was set at ratio of 80/20 in favour of the disodium hydrogen phosphate as this gave a stable buffered pH of 7.2 ± 0.2 at 25 °C when incorporated into the

formulation. The original buffer level was a total of 6 g/l but this was increased to 7 g/l to increase selection against *E. coli* and other non-target *Enterobacteriaceae*. At 6 g/l buffer, an inoculum of approximately 10^4-10^5 CFU of *E. coli* ATCC 25922 was mostly inhibited, resulting in <50 CFU growing on the agar. The increase to 7 g/l resulted in complete inhibition of *E. coli* with no negative effects to the recovery of *Salmonella*.

3.3.6 Selective components

The selectivity of the formulation is based on the classical deoxycholate/citrate system as well as incorporation of antibiotics and buffer/bile acid synergy. 8.5 g/l of tri-sodium citrate was chosen since this is the level that is used in the Hynes modification of deoxycholate citrate agar (Hynes, 1942). During extended target organism screening, the level was reduced to slightly improve recovery of weaker/slower growing *Salmonella*. This was later increased since the gain in recovery (around 5%) was disproportionate to the extra selectivity that could be achieved against non-target organisms if it was returned to 0.85 %. The bile salts No. 3 level was determined experimentally by testing the percentage recovery of target organisms as well as the suppression of non-target organisms. Bile salts No. 3 at 0.5 % resulted in good growth of many serovars tested, but weaker serovars like *S.* Gallinarum produced smaller colonies at 24 hours compared to the formulation with 0.25 %. The higher level of bile salts was more selective against other enteric organisms like *Enterobacter* spp. however, it was deemed more important to be able to detect target organisms than it is to greater suppress non-target. This was not the case in the caprylate base as the SDS, as previously mentioned reduced the selectivity towards both non-target and target organisms, meaning the bile salt concentration could be increased to 0.45% without effecting the growth of the weaker *Salmonella* serovars. The formulation also contains ox bile which is a cruder source of bile acids. The ox bile plays a key part in the selectivity as well as improving the growth and expression of enzymes required for the utilisation of the chromogenic substrates. Previous work investigating interactions between this source of ox bile and phosphate buffers showed that it caused a much greater degree of suppression against *E. coli*,

than other sources of ox bile. It is suspected that this specific source has higher levels of other minor bile fractions as well other materials that do not seem to be present in other sources examined. The previous work showed that one of the only *Enterobacteriaceae* not negatively affected by the combination was *Salmonella*. When incorporated into the formulation (along with bile salts No. 3) the formulation resulted in complete inhibition of *E. coli* ATCC 25922 inoculated at around 10^4 CFU. If the phosphate buffer was removed the same concentration of bile acids was unable to inhibit *E. coli* on its own. The phosphate buffer in the formulation without any bile salts was unable to achieve the same performance compared to what the compounds could achieve together. The ox bile (and to a lesser degree the bile salts No. 3) also play a key part in the chromogenic reaction, improving the intensity of the colour in *Salmonella*.

Novobiocin is an aminocoumarin antibiotic produced by actinomycete *Streptomyces nivens*. It binds to DNA gyrase and blocks ATPase activity (Brock, 1956). It is widely used in culture media for the isolation of *E. coli* O157, as such strains are more resistant to novobiocin than non-O157 *E. coli*. It is also sometimes used in *Salmonella* culture media since most *Salmonella* are resistant to this level of novobiocin. In culture, novobiocin is active against Gram positives as well as some Gram negatives such as *Proteus* spp. Novobiocin is not completely heat stabile and sterilisation at the standard 121 °C for 15 minutes would result in a significant loss of activity. However, since the media is sterilised by bringing to the boil it is suitable to incorporate the compound with the base prior to sterilisation. In combination with the other selective compounds novobiocin affords good selectivity against non-target *Enterobacteriaceae*, that otherwise would grow heavily on the agar and potentially crowd the plate and make it difficult to identify positive *Salmonella* isolates. The level at which the compound was incorporated into the formulation was defined by titration experiments. Alongside the other antibiotic (detailed later), novobiocin was added pre-sterilisation in 0.001 g/l increasing increments from 0.001 g/l to 0.02 g/l. The organisms detailed in Table 3.2 were used as performance markers to establish what level was optimal for performance. Quantitative recovery of the organisms was carried out using TSA as a control and 80-120 CFU as an inoculum level. The aim was to achieve good

suppression of the non-target organisms without negatively effecting the recovery of *Salmonella*. A concentration of 0.015 g/l was found to be optimal to adequately suppress non-target organisms, such as *E. aerogenes* ATCC 13048 to less than 50 % recovery compared to the control. This concentration did not reduce the recovery of the *Salmonella* in Table 3.2, whereas concentrations above 0.015 g/l did start to reduce the recovery percentage of the *Salmonella* tested. Once incorporated in the media the compound seems relatively stable showing no reduction in suppression for at least 10 weeks. The main loss of activity is a result of the sterilisation conditions and length of time it is held at 47 °C to temper prior to pouring. Novobiocin at 0.015 g/l allows for losses during preparation as well as storage of the media, without majorly negatively effecting its ability to supress non-target organisms.

Cefsulodin is a third-generation cephalosporin and like other β-lactam antibiotics disrupts the synthesis of the bacterial cell wall and prevents growth. Cefsulodin is specifically active against *Pseudomonas aeruginosa. Pseudomonas* needs to be inhibited on this formulation since it can use the target chromogen but not the masking chromogen, thus growth would result in a blue colony that would be identified as *Salmonella*. Many commercially available formulations use or recommend cefsulodin for the inhibition of *Pseudomonas.* However, they all add it as a post sterilisation supplement, as it is degraded by heat. Much like the novobiocin, the level of cefsulodin was titrated in increasing amounts, but both before and after sterilisation to see if it was possible to incorporate it into the base formulation. The level of cefsulodin required to effectively inhibit *Pseudomonas* depended on when the antibiotic was added and ultimately, how long the agar plates needed to be effective for. Cefsulodin is not very heat stable, but since this formulation is sterilised by boiling, it was suspected that it was possible to incorporate it prior to sterilisation. This was done by dissolving the compound in water and filter sterilising it through a 0.2 µm syringe filter. All solutions were made fresh as required. When added after sterilisation, 1 mg/l of cefsulodin completely inhibited a streaked inoculation of around 10^6 CFU of *P. aeruginosa* ATCC 9027 & ATCC 27853. These plates were also effective at inhibiting *Pseudomonas* for at least 2 weeks if stored in the fridge. After that, *Pseudomonas* will start to break through when inoculated at high levels. The same level was added

prior to sterilisation and similarly tested. The ability was to inhibit *Pseudomonas* was variable but mostly failed to completely inhibit when inoculated as per the previous experiment. This was most likely due to the damage sustained during boiling, which leads to reduced activity of the compound. So, the compound was tested as an addition before sterilisation, but at a higher level to account for the losses incurred during boiling. It was discovered that at least 3 mg/l is required to have the same effectiveness of activity against *Pseudomonas*, as a 1 mg/l post sterilisation addition. Table 3.4 details the results of the pre-sterilisation titration experiment carried out to discover the minimum amount required to give adequate performance. *S.* Enteritidis ATCC 13076 was used as a positive marker as it had previously shown to have the greatest sensitivity to cefsulodin, from the *Salmonella* detailed in Table 3.2. This *Salmonella* was inoculated at 80-120 CFU. Both ATCC 9027 and ATCC 27853 were inoculated from a 10-fold dilution of a stationary phase culture as a 5 µl line.

Table 3.4 Performance of increasing concentrations of cefsulodin added pre-sterilisation to the chromogenic *Salmonella* agar formulation. Organism short codes detailed in Table 3.2. TNTC – too numerous to count, NG – no growth.

	1 mg/l	2 mg/l	3 mg/l	4 mg/l	5 mg/l	6 mg/l	7 mg/l	8 mg/l	TSA control count
Sale76	91	92	92	88	86	71	55	38	98
% Recovery	93%	94%	94%	90%	88%	72%	56%	39%	
Psa27	>200	57	NG	NG	NG	NG	NG	NG	TNTC
Psa53	>200	45	NG	NG	NG	NG	NG	NG	TNTC

Once poured and set the agar plates have a finite shelf life even when kept in the fridge (2-8 °C). The dry powder antibiotic is stored long term in the freezer (<-20 °C). Once hydrated the antibiotic will start to degrade and lose activity. To account for the powder being stored at 2-8 °C not <-20 °C and variable losses during preparation of the media, 5 mg/l of the compound was chosen for the final formulation. This was because it did not appear to have any negative effects on the recovery of sensitive target organisms. Also, when considering the practicalities of powder blending, it is easier to homogenise a larger amount of a crucial component. Plates were made with 5 mg/l cefsulodin and

stored at 2-8 °C and tested in the same manner as detailed in Table 3.4 every week up to 8 weeks. It was only at 7-8 weeks did *Pseudomonas* start to break through and grow. The previous experiment had showed that adding >6 mg/l cefsulodin made the plate too harsh for some weaker *Salmonella* serovars, reducing their recovery close to or less than 50 %. After storage, the plates lost some activity and recovered the weaker *Salmonella* serovars >50 % whilst still inhibiting *Pseudomonas*. However, this made the plates initially unusable, so the level was set at 5mg/L for the fully blended dry powder format, since this gave the required performance after initial pouring and after storage for up to 8 weeks at 2-8 °C.

3.3.6.1 Prepared scale considerations

Anticipating the production of a pre-prepared plates made in an automated media preparator, dosing experiments were carried out to establish the optimum level to dose cefsulodin to achieve a functioning plate with at least 8 weeks shelf life. The previous experimentation had shown that a post sterilisation addition of 1 mg/l, was sufficient to achieve inhibition of *Pseudomonas*. However, this experiment did not consider the shelf life of the prepared plate, nor the possible thermal damage incurred by holding molten agar formulation at around 47 °C during pouring. The most robust way of dosing the media was to add a freshly made filter sterilised (0.2 µm) solution of cefsulodin in deionised water after sterilisation. A titration experiment similar to the pre-sterilisation supplementation work, showed that adding >4 mg/l cefsulodin made the plate too harsh for some *Salmonella* serovars. Addition of 3 mg/l did not show this effect, so shelf life experiments were carried out at this concentration. An 8 litre batch of media was prepared in an AES S8000 media preparator. The media was sterilised at 100°C for 1 minute, before being cooled and held at 47°C during automated pouring using an AES automated pourer stacker. Novobiocin was added before sterilisation at 13 mg/l and cefsulodin was added after sterilisation once the media had cooled to 47 °C at 3 mg/l as a filter sterilised solution in deionised water. The bulk of the prepared plates were stored at 2-8 °C for 8 weeks. Some plates were stored at 30 °C after pouring for up to 4 days, with portions of them being

removed after each sequential day. This was to simulate the possible temperature damage that may be encountered during shipping of such a product. The plates were then tested for inhibition of *P. aeruginosa* ATCC 9027 & ATCC 27853 at several serial dilutions inoculated as 5µl lines, as well as recovery of *S.* Enteritidis ATCC 13076 inoculated as 50µl spirals. The first-time point was four days after manufacture to allow for all the held at 30°C plates to be inoculated at the same time. The second-time point was 5 weeks and the last was 8 weeks. The plates showed no significant loss in performance at any time point with the recovery of *S*. Enteritidis with all plates tested achieving >50% recovery. The final time point showed higher recovery of *Salmonella* than the first, but this is to be expected since the plates are less selective due to degradation of the antibiotics and thus less harsh to all organisms.

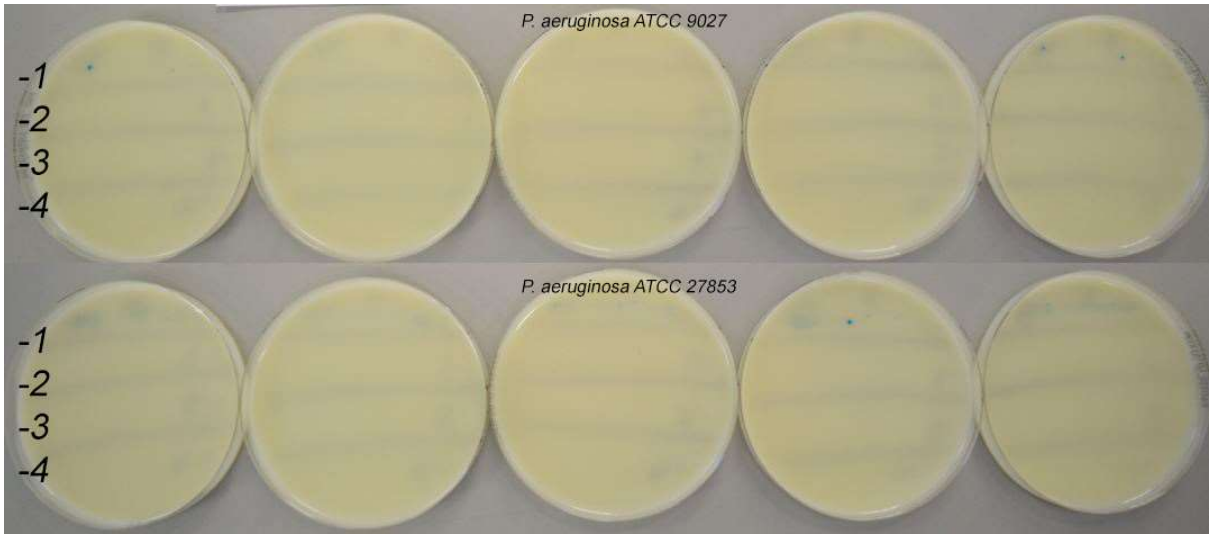


Figure 3.11 The result of two strains of *P. aeruginosa* inoculated at the first time point in the experiment detailed in section 3.3.6.2. Left to right shows plates 0-4 days stored at 30 °C prior to test. -1 to -4 denote 10-fold serial dilutions (from the preceding) from a stationary phase enrichment.

At the first time point all plates showed no growth of either *Pseudomonas* (there are 3 visible colonies that are not counted as growth due to the number of cells inoculated). This showed that even with the high thermal exposure there was still enough active antibiotic to prevent growth.

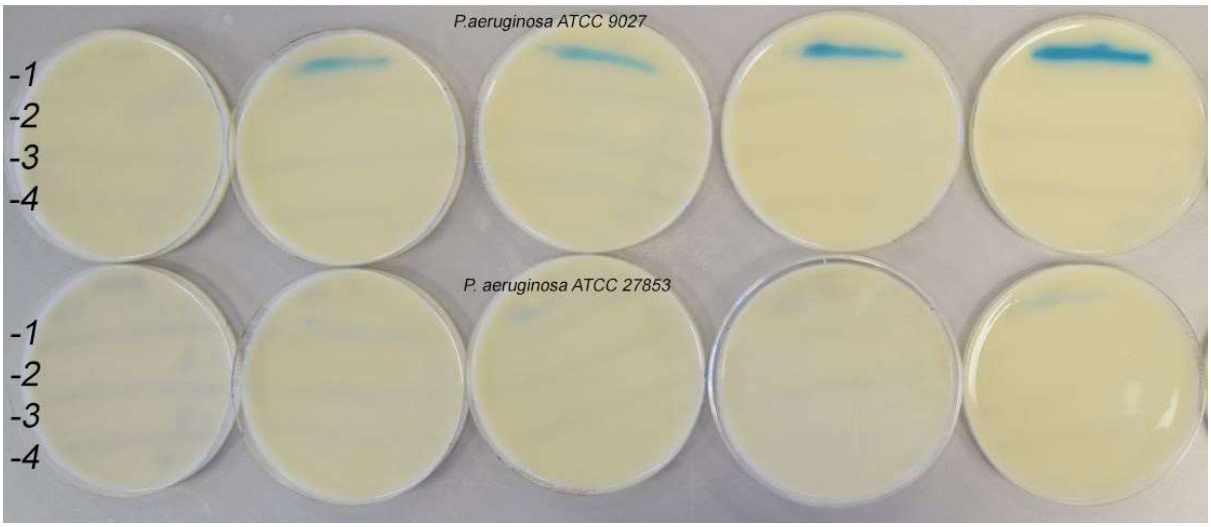


Figure 3.12 The result of two strains of *P. aeruginosa* inoculated at the 5-week time point detailed in section 3.3.6.2. Left to right shows plates 0-4 days stored at 30 °C prior to test. -1 to -4 denote 10-fold serial dilutions (from the preceding) from a stationary phase enrichment.

After 5 weeks storage at 2-8 °C the plates were tested again. This time *P. aeruginosa* ATCC 9027 started to break through at the -1 dilutions on the plates that had been kept at 30 °C. ATCC 27853 also started to partially break through but only on the plate that had been kept for 4 days at 30 °C. This level of inhibition is still very much acceptable since a -1 5 µl line corresponds to approximately $1x10^6$ cells, which is greater than what may be encountered after enrichment of foodstuff matrices. *P. aeruginosa* ATCC 9027 appears to be slightly more resistant to cefsulodin than ATCC 27853 since it can break through earlier.

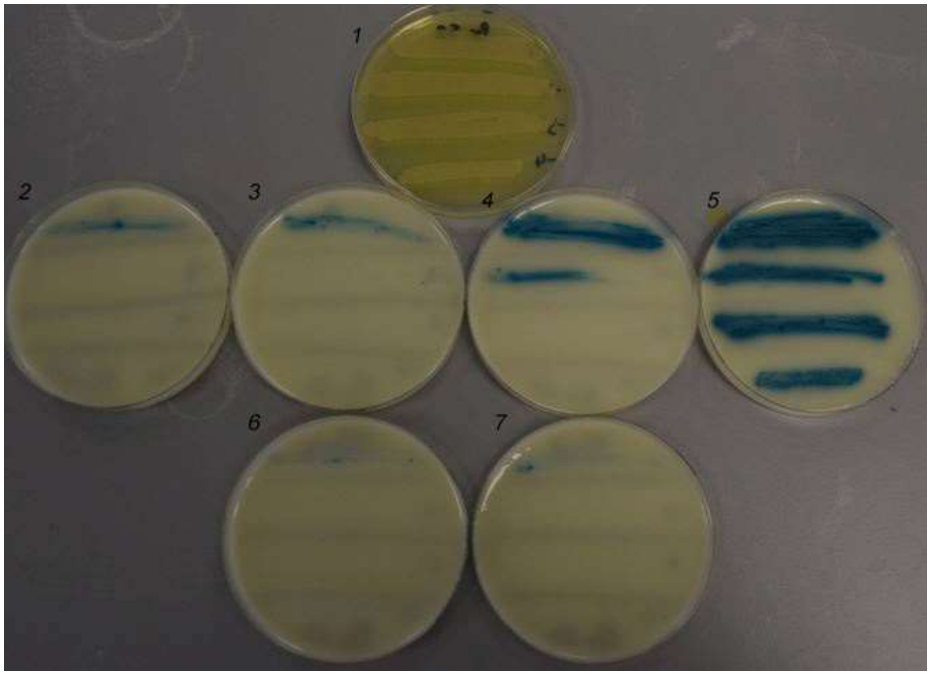


Figure 3.13 The result of *P. aeruginosa* ATCC 9027 inoculation at the 8-week time point detailed in section 3.3.6.2. Increasing serial dilutions have been inoculated as a 5 µl line from -1 at the top to -4 at the bottom of each plate. Plate 1 is a TSA control. Plates 2-5 have been stored at 30°C for 1-4 days respectively before storage for 8 weeks. Plate 6 & 7 have been stored in the fridge for 8 weeks but plate 6 is one of the first plates poured from the batch and 7 is one of the last plates poured of the batch.

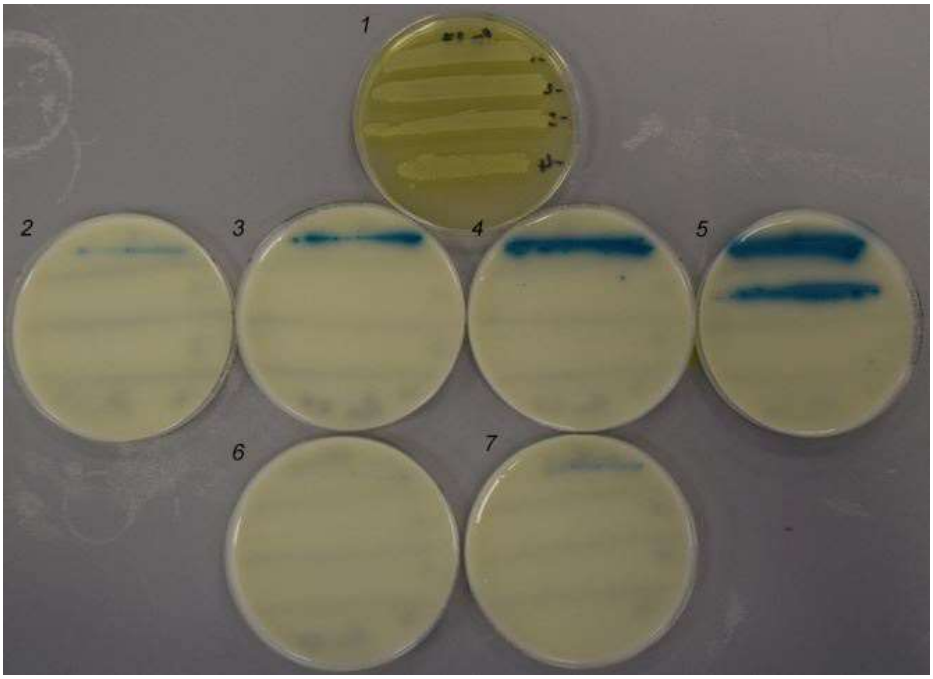


Figure 3.14 The result of *P. aeruginosa* ATCC 27853 inoculation at the 8-week time point detailed in section 3.3.6.2. Increasing serial dilutions have been inoculated as a 5 µl line from -1 at the top to -4 at the bottom of each plate. Plate 1 is a TSA control. Plates 2-5 have been stored at 30°C for 1-4 days respectively before storage for 8 weeks. Plate 6 & 7 have been stored in the fridge for 8 weeks but plate 6 is one of the first plates poured from the batch and 7 is one of the last plates poured of the batch.

The last time point at 8 weeks was the final one since ATCC 9027 breaks through heavily on plate 5 which has been temperature abused for 4 days prior to storage. The prepared agar can be kept in the fridge for >8 weeks and still be fully functional. Again ATCC 9027 seems to have a higher minimum inhibitory concentration (MIC) than ATCC 27853. The high temperature exposure plates show that the prepared agar is still functional for its 8-week shelf life even when held in relatively hot conditions for up to 3 days.

3.3.7 Agar

At various stages in development different agars were tested for the effect on the performance of the formulation. A range of European and American type agars were tested with various gelling points and

cation levels. Each different agar was tested with the organisms detailed in Table 3.2 and compared to the most commonly used European type agar, bacteriological agar No. 2. There was no major improvement from any agar over the standard bacteriological agar No. 2. Minor improvements were seen when using higher grade agars in respect to colony size and colour, but these improvements were offset by the expense and availability of these agars. A concentration of 1.25 % of agar No. 2 appeared to be suitable to yield an adequate gel strength.

3.3.8 Carbohydrates

Very early in the development of the chromogenic media it was noted that inclusion of fermentable carbohydrates was detrimental to the chromogenic reaction.

3.3.8.1 Sugar additions

To investigate the role of different sugars, a modified version of DCA (LAB065, Lab M) was made excluding the neutral red, sodium thiosulphate and lactose and the base was then supplemented with four different combinations of carbohydrates, as detailed in Table 3.5.

Table 3.5 The modified DCA formulation and carbohydrates used in the sugar additions experiment, detailed in 3.3.8.1.

Material	g/l
Beef Extract	3
Meat Peptone	2.5
Tryptone	2.5
Sodium Deoxycholate	3.5
Tri Sodium Citrate	5.7
Magnesium Chloride	0.5
Ox Bile	1
Yeast Extract	2
Sodium Pyruvate	0.5
Tris Base	0.2
Agar No. 2	12
Magenta Caprylate	0.3

Additions	
1	10 g/l xylose
2	10 g/l sucralose
3	10 g/l mannitol
4	5 g/l xylose + 5 g/l lysine

Sucralose is an artificial sugar and is trichlorinated thus is unable to be metabolised. Mannitol is a sugar alcohol and can be fermented by *Salmonella*. Lysine is an amino acid which *Salmonella* can decarboxylate and is employed along with xylose in XLD agar. All formulations were weighed into Erlenmeyer flasks and made up in 500 ml volumes. Media was sterilised by bring to the boil, cooled to 47 °C in a water bath and poured into Petri dishes.

The four plates were inoculated separately with approximately 120 CFU of *S.* Typhimurium ATCC 14028, *S*. Enteritidis ATCC 13076 and *S.* Virchow NCIMB 50077, using a 50 µl deposition from a spiral plater.

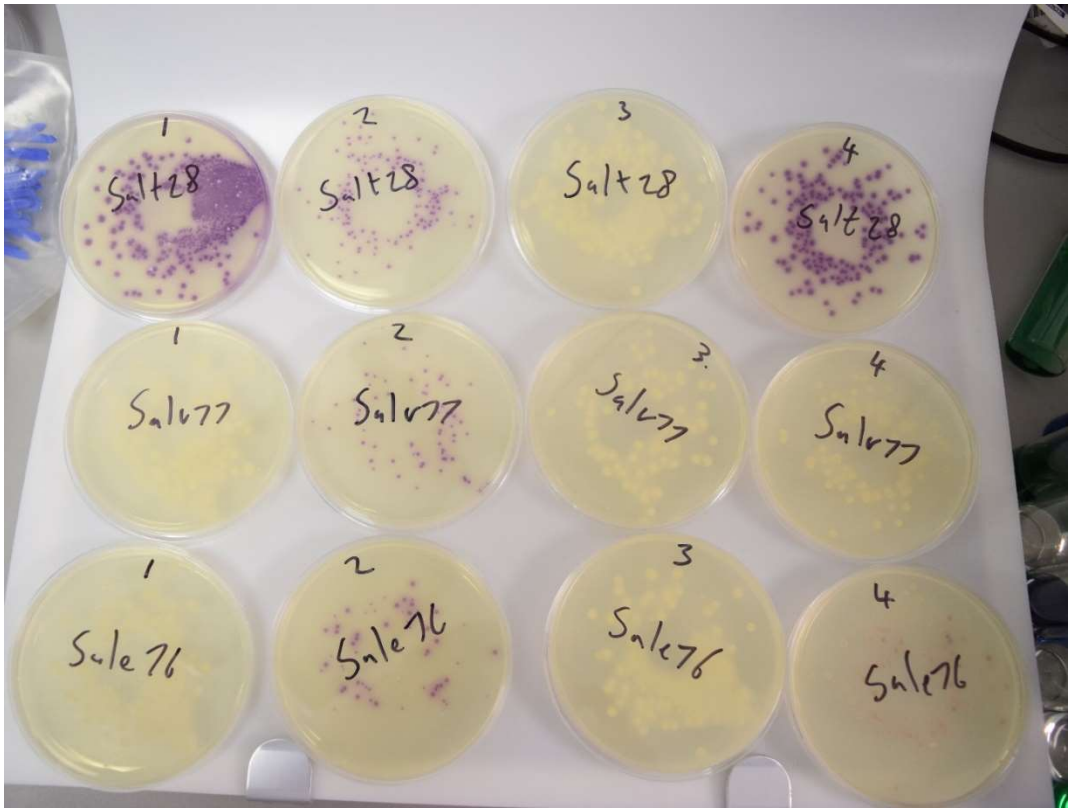


Figure 3.15 Growth and chromogenic activity of three *Salmonella enterica* serovars, on the four different sugar formulations described in Table 3.5. Salt28 is ATCC 14028, Salv77 is NCTC 5742 and Sale76 is ATCC 13076. Formulation 1 contains xylose, formulation 2 contains sucralose, formulation 3 contains mannitol and formulation 4 contains xylose and lysine.

Figure 3.15 shows there is a general pattern of poorer chromogenic activity when a fermentable carbohydrate is present for this panel of organisms. For formulation 3 when mannitol is present, there is no purple colour change, but there is good growth and recovery of the organisms. There clearly is acid production since the colonies have a fuzzy white precipitate around the colonies which represents precipitation of the bile acids. In this case it seems like exposure to the carbohydrate and/or the production of acid and local pH shift down regulates the production of esterase in this panel of organisms. Conversely on plate 2, where there is an artificial sugar which cannot be metabolised, all *Salmonella* are producing esterase and are producing purple colonies. The colonies are smaller than the other plates, but this is probably due to the selective properties of sucralose (Omran *et al*., 2013). Plates 1 and 4 show the effect of xylose at two different concentrations. On plate 1, ATCC 14028 is growing well and producing a strong chromogenic reaction, however the other two organisms are not utilising the chromogen but growing well. Plate 4 shows a similar pattern but this time ATCC 13076 is starting to use the chromogen. This suggests it is a concentration effect perhaps linked to the amount of fermentation by-products produced. Further carbohydrates were tested including glucose, maltose and rhamnose, but the same pattern of variable performance of *Salmonella* depending on their metabolism of the compound. For example, rhamnose improved ATCC 13076 but reduced the reaction in other *Salmonella*. Glucose massively reduced the activity of all *Salmonella* serovars tested but did not affect the chromogenic reaction of *Pseudomonas*. In all cases it appears that if an organism possesses the ability to cleave the chromogen, but produces acid because of fermentation, the localised pH shift either inhibits the enzyme or down regulates its production. Due to this observation of inconsistent activity when carbohydrates are included they were omitted from the base formulation.

3.3.8.2 Cellobiose

Towards the end of the development it was discovered that there was an organism that was giving a variable colour pattern, presenting a mix of green and black colonies. *Enterobacter cloacae* ATCC

13047 can use both the target and the masking chromogens, yet this organism seemed to utilise the target chromogen preferentially resulting in dark green colonies in the nonanoate base and pink/blue colonies on the caprylate base at 24 hours. Cellobiose is a disaccharide consisting of two glucose units. The masking chromogen is targeted by β-glucosidase which is the same enzyme responsible for the breakdown of cellobiose. Using this logic, a small amount (1 g/l) of cellobiose was incorporated into the medium. With this addition, after at least 20 hours incubation *E. cloacae* ATCC 13047 was fully black or blue with no hint of green or pink on either version of the chromogenic formulation. The performance of *Salmonella* did not appear to be affected with good recovery and intensity of the chromogenic reaction after 18 hours. This was not surprising since *Salmonella* should not be able to metabolise the compound. The level of cellobiose was titrated down to discover the smallest amount possible that would have the same effect. It was observed that 0.25 g/l gave a massive improvement over no cellobiose, but *E. cloacae* ATCC 13047 still having green or pink/blue hue at 18 hours incubation. Increasing the concentration to 0.5 g/l proved to be as effective as 1 g/l suggesting that this was the level that was required for sufficient induction of the glucosidase enzyme. Cellobiose is used in the formulation to force the metabolic pathway of non-target organisms, reducing the chance of false positives. The level was kept intentionally low due to the possibility of some *Salmonella* possessing the ability to ferment it and produce acid (Schafler and Mintzer, 1959). However, since the carbohydrate will be quickly exhausted at this concentration there is little risk that it can have an adverse effect on *Salmonella* esterase production.

3.3.9 Extended testing and comparison

An extended panel of organisms was tested with the nonanoate base, alongside the Conda *Salmonella* Chromogenic (SC) agar, Lab M ABC agar and XLD. Quantitative and qualitative recovery testing, using streak and spiral inoculation was performed using TSA as a control. All were prepared and sterilised by boiling, before being cooled to 47 °C and poured into Petri dishes. All plates were incubated at 37 °C for 18-24 hours. The caprylate version of the chromogenic plate was not used in this study as the nonanoate version appeared superior. Also, the commercially available product utilised the same magenta caprylate chromogen, so acted as a marker for this chromogen type.

Table 3.6 Qualitative recovery of 5 µl steaks of approximately 10^6 CFU of various bacterial test strains on the Nonanoate base, Conda *Salmonella* Chromogenic (SC) agar, Lab M ABC agar and XLD. + - growth on primary first quadrant only, ++ - growth on first and second quadrant only, ++ - growth on first to third quadrants, ++++ - growth on all quadrants, NG – no growth. Refer to materials and methods 3.2 for short code reference. Cells highlight in red indicate a false result (positive or negative).

Short Code	Nonanoate	Conda SC	ABC	XLD
Psa53	NG	+++	+++	+++
Psa27	NG	++	++	++
Citf64	NG	++++	NG	+
Citf50	+	++++	+++	++++
Prom83	NG	+++	NG	+++
Prom38	+	+++	+++	+++
Prom90	NG	+++	+++	+++
Ec22	NG	+	++	1/2+
Ec39	NG	+++	+++	++
Shso30	+	++	+++	+++
Shso74	NG	++	+++	+++
Shfl22	++	+++	+++	+++
Sa23	NG	NG	NG	NG
Entcfs12	NG	NG	NG	NG

Table 3.7 Quantitative counts (expressed as colony forming units) and percentage recovery of various bacterial test strains on the Nonanoate base, Conda *Salmonella* Chromogenic (SC) agar, Lab M ABC agar and XLD. Refer to materials and methods 3.2 for short code reference. Cells highlight in red indicate a false result (positive or negative).

	Nonanoate		Conda SC		ABC		XLD		TSA
Short Code	Count	%	Count	%	Count	%	Count	%	Count
L1	55	80%	56	81%	57	83%	44	64%	69
L2	90	98%	95	103%	80	87%	88	96%	92
L3	48	91%	63	119%	52	98%	63	119%	53
L4	77	120%	51	80%	58	91%	50	78%	64
L5	48	96%	53	106%	66	132%	49	98%	50
L6	53	106%	59	118%	50	100%	46	92%	50
L7	90	82%	89	81%	85	77%	95	86%	110
L8	54	79%	62	91%	68	100%	55	81%	68
L9	41	132%	0	0%	28	90%	37	119%	31
L10	72	111%	56	86%	76	117%	62	95%	65
L11	47	80%	52	88%	38	64%	44	75%	59
L12	65	90%	54	75%	68	94%	60	83%	72
L13	39	64%	60	98%	45	74%	61	100%	61
L14	65	110%	69	117%	60	102%	59	100%	59
L15	64	79%	106	131%	57	70%	60	74%	81
Salt28	87	104%	91	108%	107	127%	87	104%	84
Sala17	88	97%	75	82%	84	92%	81	89%	91
Sale76	45	82%	51	93%	42	76%	43	78%	55
Salv42	58	107%	47	87%	47	87%	39	72%	54
Salar55	73	106%	63	91%	66	96%	76	110%	69
Sald74	109	111%	106	108%	99	101%	102	104%	98
Salpo40	72	104%	89	129%	60	87%	65	94%	69
Entba48	17	31%	63	115%	52	95%	44	80%	55
Entbaco5	11	10%	102	93%	101	92%	104	95%	110
Cs20	0	0%	41	98%	37	88%	35	83%	42

The extended panel of organisms revealed no issue with the nonanoate formulation regarding sensitivity, specificity and selectivity. As expected, false positives and negatives were observed with the C8 esterase based chromogenic plate from Conda. ABC yielded false negative results for isolates L8, L9 & L15 which all appeared to lack α-galactosidase, and thus grew colourless. XLD only suffered a false negative reaction with *S.* Gallinarum which does not produce a positive H_2S reaction. However, XLD was the least selective of all the media tested, yielding on average the most non-target colonies.

3.3.10 Commercial product comparison

To test the relative performance of the two chromogenic formulations a commercially available, ready prepared *Salmonella* chromogenic esterase agar was purchased to compare against. In the UK one of the most widely used *Salmonella* chromogenic agars is the Brilliance *Salmonella* agar (PO5098) by Oxoid Ltd (Thermo Scientific). The full panel of *Salmonella* detailed in Table 3.1 and 3.2 were streaked onto the agars from a dilution in MRD of a stationary phase overnight culture in TSB to yield approximately 10^4-10^5 CFU. A 10 µl loop was used to inoculate the agars, which was divided into 3 sections. Plates were then incubated for 37 °C for 24 hours and observed, with special attention paid to the level of growth and colour judged by eye. Qualitative recovery was judged by how far along the streak the organisms had grown, and colour intensity was judged in comparison to the strongest intensity observed.

Table 3.8 Qualitative growth response of the *Salmonella* test panel on both the caprylate and nonanoate formulations compared to the Oxoid product. Colony colour is typical unless marked asterixis, where by atypical colour was observed.

	Nonanoate	Caprylate	Oxoid Brilliance Salmonella
S. Typhimurium ATCC 14028	+++	+++	+++
S. Enteritidis ATCC 13076	+++	+++	+++
S. Virchow NCIMB 50077	++	+++	+++
S. Poona NCTC 4840	+++	+++	+++
S. Arizonae NCTC 7355	+++	+++	+++
S. Dublin ATCC 39184	+++	+++*	+++*
L1: 501249 DT193 *4,*[5],*12*:i:-	+++	+++	+++
L2: *S.* Enteritidis 01-00493-2	+++	+++	+++
L3: 500360 U288 *4,*[5],*12*:i:-	++	+++	+++
L4: SARB18 FB *S.* Enteritidis	+++	+++	+++
L5: 000398ST DT193	+++	+++	+++
L6: 500343 STU288	+++	+++*	+++*
L7: ST F98	+++	+++	+++
L8: ST D23580	+++	+++	+++
L9: *S.* Gallinarum 287/91	+++	+++	No Growth
L10: *S.* Typhimurium DT193	+++	+++	+++
L11: *S.*E P1251D9	+++	++	+
L12: ST 244	+++	+++	+++
L13: ST04327 DT194 *4,*[5],*12*:i:-	++	++	+++
L14: ST 4/74	+++	+++	No Growth
L15: *S.* Arizonae (ONPG positive)	++	+++	+

Key		
	*	Atypical colouration (false negative)
	+	Growth up to the first streak section
	++	Growth up to the second streak section
	+++	Growth up to the third streak section

Table 3.8 demonstrates the common reported problems with the current generation of esterase based chromogenic *Salmonella* agars. Both the Oxoid and caprylate formulation target C8 esterase and both suffer the same issue with weak chromogenic reactions for some *Salmonella* like *S.* Dublin Also, the L6 isolate displayed the same issue, with poor to no positive colour production. The Oxoid product also failed to grow a *S.* Gallinarum which is representative of a non-motile serovar. This is most likely due to the selectivity employed in that formulation not the chromogen used as the

caprylate base grew this organism well. Organisms like *S.* Dublin did not suffer with poor utilisation of the chromogen on the nonanoate base and gave strong colour change.

3.3.11 Third party evaluation

Association Française de Normalisation (AFNOR) is a French 3rd party certification body and the member body for France of ISO. They award certification of alternative methods to those stated in the ISO standards. ADRIA development is an expert laboratory, again in France that can be used to carry out studies that contribute towards the validation of alternative methods against a reference method according to ISO 16140-2. In this case, the reference method is the horizontal method for Salmonella detection, ISO 6579. Both formulations were included in an alternative method study conducted by Neogen Europe at ADRIA development. This opportunity allowed both media formulations to be tested against the ISO method utilising a popular C8 esterase chromogenic plate, ASAP (BioMerieux). Various matrices were tested in the unpaired study with both artificial and naturally contaminated samples. There was also 5 x pooling alongside single analysis, where 5 original buffered peptone water (BPW) enrichments were mixed together before plating. This was an attempt to validate a method that would allow end users to pool samples effectively saving resources and time. The individual samples would only be tested if the pooled sample gave a positive result. The methodology included the use of a selective supplement provided by Neogen Europe to add to BPW so that it could be used as a one-step enrichment for a molecular platform alongside the chromogenic agar. Each sample was also subcultured into Rappaport-Vassiliadis Soya Peptone Broth (RVS) as per the ISO method to assess the present of *Salmonella* even if the one step enrichment gave a negative result. This was because artificial spiking was carried out with stressed (48 hours at 2-8 °C held on the matrix surface) *Salmonella* (various spp.) dosed at an average of around 3 CFU per 25g of sample matrix. With such low-level inoculation, it is possible that some samples are not actually inoculated with any viable cells. A secondary enrichment is used to allow time for further multiplication in case the original enrichment did not reach a detectable level. The concept is that if after secondary enrichment no growth is seen,

it is likely the original sample was not inoculated with any viable cells. The results were compared against the standard ISO 6579 protocol and were reported as negative agreement (NA) or positive agreement (PA), where both methods reported the same negative or positive result. Or as negative disagreement (ND) where the ISO method did detect the target, but the alternative did not, or finally as positive disagreement (PA) where the ISO method failed to detect the target but the alternative did. The ratio of disagreements is essentially how an alternative method is validated against the standard method. If ND≤PD the method will pass the ISO 16140-2 validation, if ND≥PD the methodology will not pass.

Table 3.9 The positive and negative deviations of both the caprylate and nonanoate formulations when used with a single stage or dual stage enrichment protocol in both individual or pooled test, compared to the ISO 6579 standard.

	Single Enrichment				Secondary Enrichment			
	Caprylate		Nonanoate		Caprylate		Nonanoate	
	ND	**PD**	**ND**	**PD**	**ND**	**PD**	**ND**	**PD**
Individual	6	3	5	3	3	7	3	6
Pooling	6	3	6	3	3	7	4	8

Table 3.9 shows that there was no difference between either chromogenic base in any test. The single enrichment protocol fails the validation since ND>PD, but when the samples are put through secondary enrichment in RVS the methodology exceeds that of the ISO since PD>ND. This result means that the single step enrichment is unsuitable when paired with the protocol designed by Neogen Europe, but the agar plates themselves are performing well. The single stage protocol with selective supplement would most likely have performed well compared to the ISO standard, if the inoculation level of the target organism was higher. It was commented that the nonanoate plate was easier to read as the colonies were easier to differentiate compared to the caprylate base. Also, there was

generally less non-target growth on the nonanoate plate due to superior selectivity. With this information, it was decided that the caprylate formulation should be dropped as it did not reach all the intended design goals and only the nonanoate formulation was taken forward. The nonanoate formulation was also novel as this pairing of chromogenic substrates had not been described before. The formulation was given the name Chromogenic Agar for Salmonella Esterase (CASE).

3.3.12 Commercial laboratory testing

CASE was sent to several of the major UK food laboratories that carry out *Salmonella* testing. All sites followed the ISO 6579 methodology and used XLD and Brilliant Green Agar (BGA) as final plating media. All sites used CASE as an alternative plating medium alongside their current method for testing foodstuffs (typically ready to eat meal, raw meat and other various processed foods).

3.3.12.1 ALcontrol (now ALS)

Four sites across the UK trialled the media; Dunstable, Newton Abbott, Rotherham and Shrewsbury.

Dunstable

115 separate samples (including controls) of different matrices were tested with their current method plus CASE. All samples gave concurrent results (detecting *Salmonella* in 26 samples), except for one sample spiked with *Citrobacter braakii*.

Figure 3.16 Citrobacter *braakii* streaked on BGA (left) XLD (middle) and CASE (right).

Citrobacter braakii is known to present false positive results on traditional media as seen in Figure 3.16. On CASE, due to strong glucosidase activity the organisms give a true negative result with black colonies.

Newton Abbott

180 separate samples (including controls) of different matrices were tested with their current method plus CASE. No sample yielded a positive *Salmonella* isolate except for the positive controls. However, there was serval occasions where XLD & BGA both presented false positives where CASE did not. These organisms were identified by Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometry, referenced to the in-house database. Common false positives belonged to the following organisms; *Hafnia alvei*, *Citrobacter gillenii* and *Citrobacter braakii.* There was a single incidence of CASE resulting in a false positive caused by *Pantoea calida* isolate where this organism presented both black and green colonies. Some isolates of *Enterobacter cloacae* and *Proteus hauseri* also presented colonies with a slight green hue to them (see Figure 3.17), but were easily distinguished from true positives and were not misinterpreted.

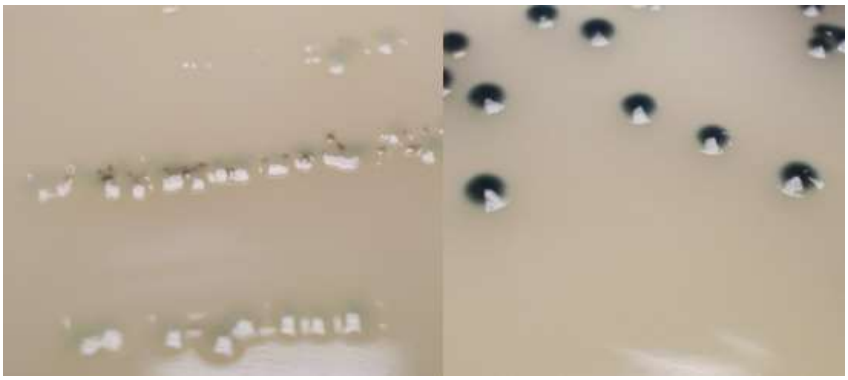


Figure 3.17 Isolates of *Proteus hauseri* (left) and *Enterobacter cloacae* (right) on CASE after 24 hours incubation at 37 °C.

The site also tested atypical, lactose fermenting *Salmonella* they had previously encountered and failed to initially positively identify with their current method. These isolates gave strong positives on CASE (see Figure 3.18).

Figure 3.18 A lactose fermenting *Salmonella* isolate on XLD (top right), BGA (bottom right) and CASE (top & bottom left).

The site also tested if there was any difference in performances between pre-prepared media and in house prepared DCM. No difference was observed.

Rotherham

45 matrices samples were processed alongside their current method. No naturally contaminated *Salmonella* isolates were observed on any of the agars. There were three incidences of isolates of *Klebsiella pneumoniae* (identified by MALDI-TOF) presenting dark green/black colonies on CASE. These isolates yielded negative results when further testing was carried out. The site also noted no difference between pre-prepared media and in house prepared DCM.

Shewsbury

100 separate samples (including controls) of different matrices were tested with their current method plus CASE. Three naturally contaminated *Salmonella* isolates were identified on all agars. There was a single incidence of *Citrobacter youngae* (confirmed by API, bioMerieux) yielding a false positive on XLD and BGA, but gave a strong negative black reaction on CASE.

3.3.12.2 Eurofins Scientific

Two sites were trialled; Acton and Grimsby.

Acton

100 separate samples (including controls) of different food matrices were tested with their current method plus CASE. Out of 100 samples there were 26 positive *Salmonella* and 74 negatives. XLD yielded 2 false negatives, 4 false positives and 94 true results. BGA yielded 2 false negatives, 22 false positives and 76 true results. CASE yielded no false negatives, 19 false positives and 81 true results.

Grimsby

6 matrices samples were processed alongside their current method. There were 2 true positives and 4 true negatives recorded on XLD, BGA and CASE. General feedback was that CASE resulted in fewer non-target colonies compared to XLD and BGA, and that positive colonies were easier to identify on CASE compared to the other agars.

Other tests sites around the UK reported similar results. Those using XLD and BGA as plating media reported an improvement in specificity and selectivity with CASE, compared to their current methodology. Those using chromogenic products reported an improvement in detection of weak esterase producing organisms, as well as improved differentiation between positive and negative colonies as demonstrated in Figure 3.19.

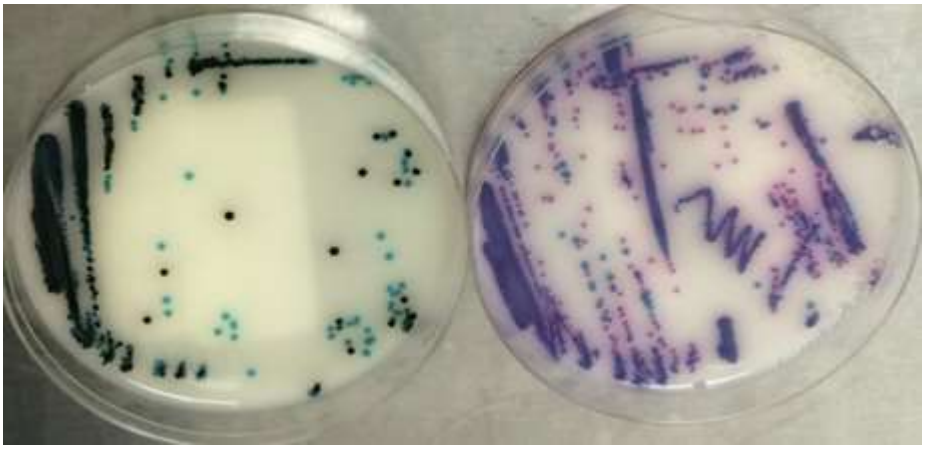


Figure 3.19 A mixed culture of *Salmonella* and non-target streaked onto CASE (left) and Oxoid Brilliance *Salmonella* Agar (right).

3.3.13 External comparison to traditional media

CASE was given to Dr Wenner of the Hinton Lab in the Institute of Integrative Biology at the University of Liverpool. They were testing environmental *Salmonella* isolates from the Liverpool School of Tropical Medicine (LSTM) and were having problems with XLD as it was yielding both false positives and false negatives. Thus, on occasion they were missing *Salmonella* and wasting time and resource in further confirming and finally sequencing what they thought was *Salmonella*. The agar was used in parallel alongside XLD during their study.

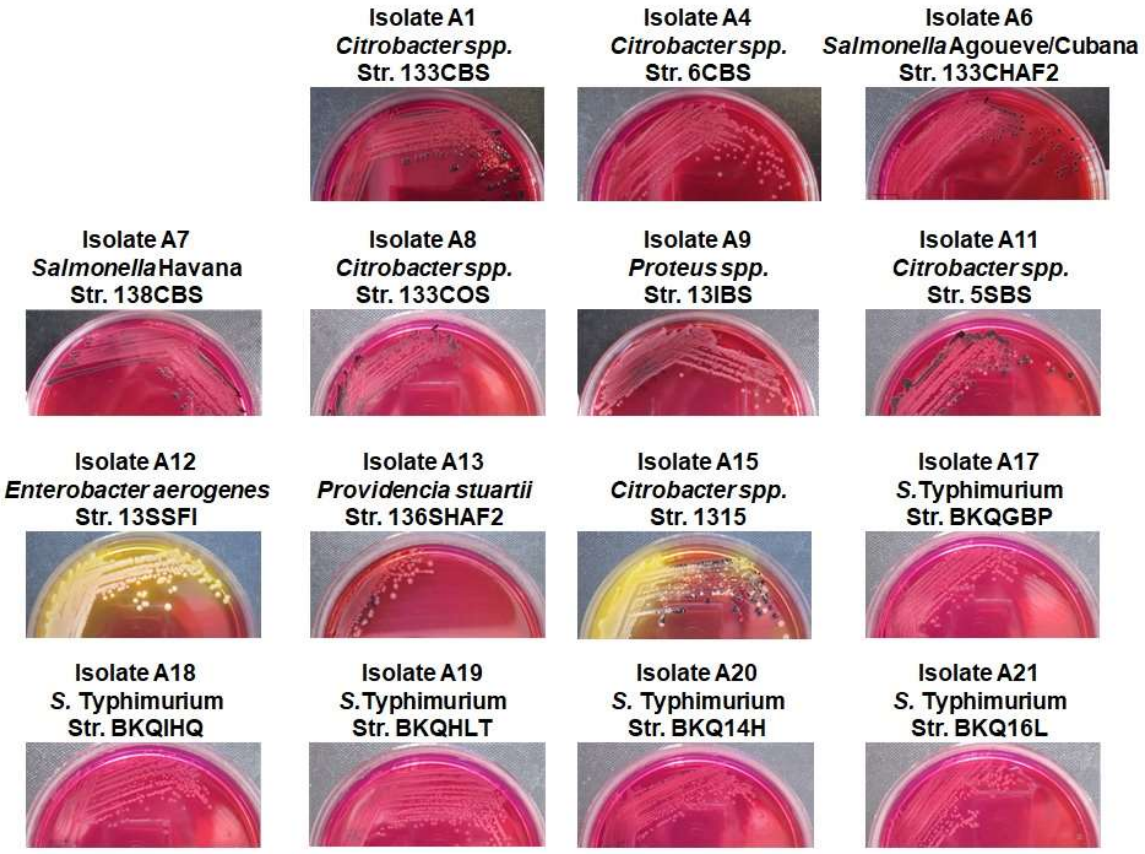


Figure 3.20A A panel of target and non-target organisms on XLD after 24 hours incubation at 37 °C.

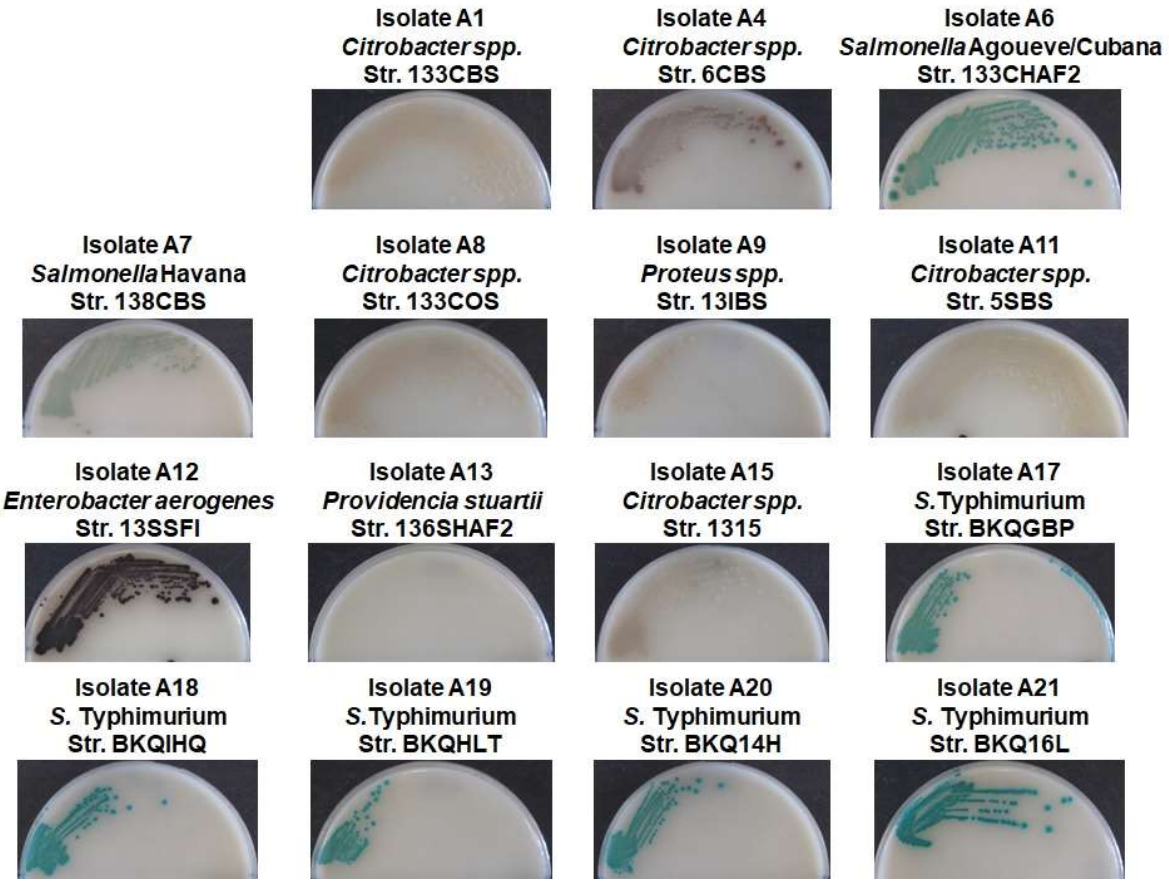


Figure 3.20B The same panel of organisms displayed in Figure 3.20A on CASE after 24 hours incubation at 37 °C.

Figures 3.20A and 3.20B show the improvement observed by the Hinton lab group in detection of *Salmonella* when using CASE compared to XLD. On XLD there was a range of *S.* Typhimurium isolates that were unable to produce H_2S meaning they developed colourless colonies resulting in incorrect identification. On CASE, these organisms all gave a strong blue/green reaction allowing for correct identification. The other improvement was the greater inhibition of non-target organisms like *Citrobacter* spp. and *Proteus* spp. This reduced the time wasted on further confirmatory testing of non-target organisms.

3.3.14 Inclusivity testing

Data was compiled from various sources to demonstrate the inclusivity of CASE. These include 3rd party trial sites such as ADRIA, as well as in house testing. Table 3.10 lists some of the recorded serovars that demonstrated a positive reaction on CASE.

Table 3.10 A list of recorded *Salmonella* that gave the expected blue/green colour and good growth on CASE.

Salmonella enterica subsp. e*nterica*		Source	Reference	Growth	Colony Appearance On CASE
1	serovar Abony	Type Culture	NCTC 6017	+	Blue/Green Colonies
2	serovar Amsterdam	Wild Type	Ad1767	+	Blue/Green Colonies
3	serovar Anatum	Wild Type	Ad2727	+	Blue/Green Colonies
4	serovar Anatum	Wild Type	Ad1108	+	Blue/Green Colonies
5	serovar Anatum	Wild Type	Ad1451	+	Blue/Green Colonies
6	serovar Arizonae	Wild Type	L15	+	Blue/Green Colonies
7	serovar Arizonae	Type Culture	NCTC 7355	+	Blue/Green Colonies
8	serovar Braendenburg	Wild Type	Ad2420	+	Blue/Green Colonies
9	serovar Bredeney	Wild Type	Ad2042	+	Blue/Green Colonies
10	serovar Derby	Wild Type	Ad1337	+	Blue/Green Colonies
11	serovar Derby	Wild Type	Ad1093	+	Blue/Green Colonies
12	serovar Derby	Wild Type	Ad1337	+	Blue/Green Colonies
13	serovar Dublin	Wild Type	Sald74	+	Blue/Green Colonies
14	serovar Enteritidis	Wild Type	01-00493-2	+	Blue/Green Colonies
15	serovar Enteritidis	Wild Type	SARB18 FB	+	Blue/Green Colonies
16	serovar Enteritidis	Wild Type	465	+	Blue/Green Colonies

Salmonella enterica subsp. e*nterica*		Source	Reference	Growth	Colony Appearance On CASE
17	serovar Enteritidis	Wild Type	2532	+	Blue/Green Colonies
18	serovar Enteritidis	Wild Type	Ad477	+	Blue/Green Colonies
19	serovar Enteritidis	Wild Type	Ad2295	+	Blue/Green Colonies
20	serovar Enteritidis	Type Culture	NCTC 13076	+	Blue/Green Colonies
21	serovar Gallinarum	Wild Type	287/91	+	Blue/Green Colonies
22	serovar Hadar	Type Culture	NCIMB 13036	+	Blue/Green Colonies
23	serovar Havana	Wild Type	Ad2728	+	Blue/Green Colonies
24	serovar Havana	Wild Type	Ad930	+	Blue/Green Colonies
25	serovar Indiana	Wild Type	2	+	Blue/Green Colonies
26	serovar Infantis	Wild Type	Ad1646	+	Blue/Green Colonies
27	serovar Infantis	Wild Type	Ad2712	+	Blue/Green Colonies
28	serovar Infantis	Type Culture	NCIMB 13036	+	Blue/Green Colonies
29	serovar Kedougou	Wild Type	Ad2419	+	Blue/Green Colonies
30	serovar Livingstone	Wild Type	F104	+	Blue/Green Colonies
31	serovar Livingstone	Wild Type	Ad2566	+	Blue/Green Colonies
32	serovar London	Wild Type	326	+	Blue/Green Colonies
33	serovar London	Wild Type	Ad1874	+	Blue/Green Colonies
34	serovar Mbandaka	Wild Type	Ad914	+	Blue/Green Colonies
35	serovar Mbandaka	Wild Type	Ad2296	+	Blue/Green Colonies
36	serovar Mbandaka	Wild Type	Ad1722	+	Blue/Green Colonies
37	serovar Meleagridis	Wild Type	505	+	Blue/Green Colonies
38	serovar Menston	Wild Type	Ad2729	+	Blue/Green Colonies
39	serovar Montevideo	Wild Type	Ad2421	+	Blue/Green Colonies
40	serovar Montevideo	Wild Type	Ad912	+	Blue/Green Colonies

Salmonella enterica subsp. e*nterica*		Source	Reference	Growth	Colony Appearance On CASE
41	serovar Nottingham	Wild Type		+	Blue/Green Colonies
42	serovar Ohio	Wild Type	Ad2213	+	Blue/Green Colonies
43	serovar Ouakam	Wild Type	Ad1647	+	Blue/Green Colonies
44	serovar Poona	Type Culture	NCTC 4840	+	Blue/Green Colonies
45	serovar Regent	Wild Type	328	+	Blue/Green Colonies
46	serovar Saintpaul	Wild Type	F31	+	Blue/Green Colonies
47	serovar Senftenberg	Wild Type	6	+	Blue/Green Colonies
48	serovar Typhimurium	Wild Type	U288	+	Blue/Green Colonies
49	serovar Typhimurium	Wild Type	ST 244	+	Blue/Green Colonies
50	serovar Typhimurium	Wild Type	ST 474	+	Blue/Green Colonies
51	serovar Typhimurium	Wild Type	ST F96	+	Blue/Green Colonies
52	serovar Typhimurium	Wild Type	Ad1335	+	Blue/Green Colonies
53	serovar Typhimurium	Wild Type	Ad1484	+	Blue/Green Colonies
54	serovar Typhimurium	Type Culture	ATCC 14028	+	Blue/Green Colonies
55	serovar Typhimurium DT193	Wild Type	000398ST	+	Blue/Green Colonies
56	serovar Typhimurium DT194	Wild Type	ST04327	+	Blue/Green Colonies
57	serovar Urbana	Wild Type	Ad2334	+	Blue/Green Colonies
58	serovar Virchow	Type Culture	NCIMB 50077	+	Blue/Green Colonies
59	serovar Virchow	Wild Type	F276	+	Blue/Green Colonies
60	serovar Virchow	Wild Type	Ad2569	+	Blue/Green Colonies
61	serovar Wandsworth	Wild Type	Ad2335	+	Blue/Green Colonies

3.3.15 False positive organism screen

Feedback from end users and experts suggested that there were several organisms that often gave atypical results on similar chromogenic agars. *Serratia marcescens* and *Aeromonas hydrophila* were two organism that were often referred cited as organisms that gave false positive reactions, as in they appeared the same colour as *Salmonella.* Two type strains were selected and streaked onto CASE at a relatively high inoculum level (>10^6 CFU). Figure 3.21 shows the result of the inoculation.

Figure 3.21 Serratia *marcescens* ATCC 274 (left) and *Aeromonas hydrophila* NCTC 8049 (right) after 24 hours incubation at 37 °C on CASE.

Neither organism grew well, with ATCC 274 producing small black colonies and NCTC 8049 producing no growth. Since *Aeromonas* is known to have strong esterase activity the test was repeated several times with increasing inoculum levels. When the inoculation level reached approximately 10^7 the plates yielded 1-10 CFU that were bright blue. The organism can use the substrate but struggles to grow on the agar due to the selectivity employed.

3.3.16 False Negative Result

During a 3rd party validation test at Marshfield Labs in Wisconsin, USA an atypical *Salmonella* isolate was detected on CASE. The sample presented colourless colonies on CASE but was positive on other *Salmonella* diagnostic agar. The isolate was tested through NeoSeek, Neogen's 16s metagenomics bacterial identification facilities.

Test : NeoSeek Salmonella Status : Ready

Animal ID	Predicted Serotype	Confidence Score	Detected_O-antg	Detected_FliC	Detected_FljB
Marshfield Salmonella	mbandaka	1.2317034	O7	z10	e_n_z15

Figure 3.22 NeoSeek result for the atypical *Salmonella* isolate described in 3.3.16.

Figure 3.22 displays the result indicating that the predicted serotype was *S*. Mbandaka. The isolate was tested on a variety of agar formulations including CASE, ABC (which targets α-galactosidase) and Columbia blood agar as a control.

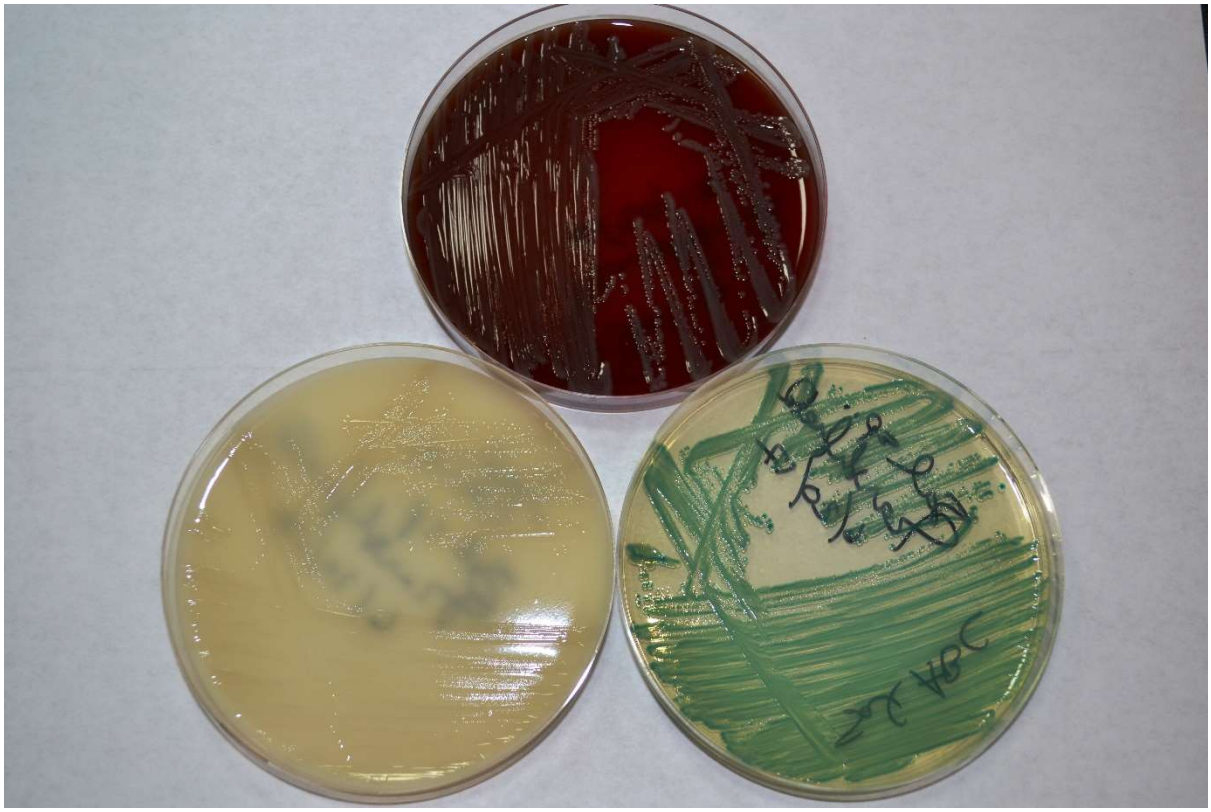


Figure 3.23 *S.* Mbandaka isolate on CASE (bottom left), ABC (bottom right) and Columbia blood agar (top)

The original laboratories observation of good growth but colourless colonies was replicated as shown in Figure 3.23. The isolate gave a strong positive reaction on ABC indicating α-galactosidase activity without detectable β-galactosidase. The isolate was also tested on the caprylate version of the

chromogenic base and yielded no chromogenic reaction. Cellobiose was also removed from the formulation and retested but had no effect on the appearance of this isolate.

3.4 Discussion

The success of the new chromogenic agar, CASE, is due mainly to two factors; the esterase substrate and the selectivity achieved by the formulation. The nonanoate chromogen appears to be vastly superior to the caprylate version for several reasons. Firstly, it demonstrated greater stability, as shown by the absence of non-specific cleavage in agar. Secondly *S.* Dublin isolates that traditionally present a weak chromogenic reaction with C8 esterase substrates, give a strong reaction with the C9 nonanoate substrate. This is most likely due to either the enzymes affinity for this substrate or perhaps for the auto inducing properties of the nonanoate. Some chromogenic substrates do not induce a good response in culture media when present on their own. This is because not enough enzyme is produced in the incubation window to generate a strong colour reaction. These substrates require what is called an inducer to encourage the target microorganism/s to produce more enzymes by up regulation. A good example is the induction of β-galactosidase to improve the rate of action upon β-galactoside substrates. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is often used as an inducer as it is an analogue of the intended substrate that cannot be hydrolysed so is not consumed, but does promote the production of β-galactosidase due to its structure. Ox bile and bile acid fraction induce the production of esterase in *Salmonella* in a similar manner. However, this is not sufficient for isolates of *S.* Dublin when using magenta-caprylate as a substrate. X-nonanoate appears to either autoinduce the required activity or is more easily cleaved by the produced esterase's of most *Salmonella enterica* serovars. X-nonanoate is clearly superior to magenta-caprylate. It appears that the only reason why it is not more widely used is partly tradition, but mainly due to the lack of availability of an adequate masking chromogen. CHE chromogens are only currently available to Lab M Ltd due to patent protection. The black masking properties, low toxicity and good reaction make it ideal for this application. This dual system has shown to be more sensitive specific when compared to the currently

commonly used pairings. Also, the clear difference between the black and blue/green make it more suitable for identification, when there is a high number of colonies present. The selectivity of the agar is also crucial to its success. Many available formulations are highly selective against non-target organisms, but what is not always taken into consideration is the growth and recovery of weaker target organisms. The development of the finely tuned selective system, was in a large part due to the availability of a wide variety of *Salmonella* isolates. The *S.* Gallinarum from the University of Liverpool proved to be an excellent marker for selective component titration. If this organism could grow well and produce a positive chromogenic reaction within 24 hours, it meant that the selective pressure would mostly likely be suitable for all *Salmonella* isolates one is likely to encounter in foodstuffs. The base formulation was also used in a custom agar developed for direct plating of heavily contaminated sample (see Appendix 1).

The 3rd party testing of the product yielded positive information regarding the performance of the formulation when following common protocols, such as ISO 6579. However, since most trial sites were using a combination of XLD and BGA it was not surprising that a chromogenic formulation could outperform their current protocol. The ISO 16140 validation study, whilst not completely successful, demonstrated the superior performance of the formulation. Here a full ISO method, including primary and secondary enrichment, followed by dual plating on XLD and a popular chromogenic was compared to a single selective enrichment followed by plating on CASE. The one stage enrichment failed to achieve a pass result due to the over selective nature of the paired broth supplement. This meant that a low (~1CFU) inoculation of stressed *Salmonella*, was in some cases not reaching a level where a single colony could be detected on CASE. But when the same samples were subculture into RVS for secondary enrichment, the positive deviations were much greater than the negative deviations. It is also worth noting that the negative deviations can be due to failure to inoculate the original test sample. This study was not a paired study, in that the same sample was not split to both the ISO and alternative method. Since the target inoculation is ~1 CFU it is possible that one sample could receive

a single cell and the other receive none. This phenomenon affects both methods equally, but with a large enough sample size, does not cause bias and yields sensible results.

The single recorded incidence of a false negative on CASE is not surprising because of the large amount of serovars and their genetic diversity. This isolate appears to lack the ability to produce a chromogenic reaction on esterase substrate-based plates, yielding no colour change on either the caprylate or nonanoate based plate. The organism grew well and produced typically sized colonies, indicating that the formulation was not too selective or lacked growth requirements of the isolate. During the ISO 16140 trial a *S*. Mbandaka serovar was used, which gave a typical reaction on CASE thus it does not appear to be a common feature of this serovar. The conclusion is that this specific isolate is a mutant without the ability to cleave esterase substrates, either due to the lack of the enzyme or radically different enzyme structure which is incompatible with the substrates.

All the initial design goals were met including performance and practicality. The new media formulation can detect H2S negative and non-motile *Salmonella,* such as *S.* Gallinarum and weak esterase producting *Salmonella*, such as *S*. Dublin. The formulation can be presented as a powdered DCM format or a prepared media. Considerations of stability and shelf life appear to date to be successful, as at time of writing real time stability of the DCM version is achieving at least 18 months (data not shown). The agar formulation is relatively expensive due to the compounds utilised but is comparable to others in the market as well as offering superior performance. The new media formulation utilises an underused esterase substrate and a previously unused (in this combination), black masking glucosidase substrate. The combination of the two utilised chromogens results in an agar formulation which is superior to other described formulations, both for the detection of *Salmonella* spp., in the presence of high non-target microorganisms.

Chapter Four

Salmonella Enrichment

4.1 Introduction

Enrichment of *Salmonella* in a foodstuff is critical for the successful detection of the pathogen. This is because levels of potential contamination in foodstuffs are likely to be far lower than what is possible for a diagnostic like an agar plate, to detect on its own. Thus, enrichment facilitates the multiplication of the target organism to detectable levels, by providing an environment that is favourable for growth. In Europe and especially the UK, buffered peptone water (BPW) is the standard for primary enrichment of *Salmonella* from foodstuffs. It provides a source of nitrogen and carbon from digests of protein sources, as well as a buffered and osmotically control environment to prevent cell damage. Due to its low cost, simplicity and wide spread availability it is also gaining traction as the primary broth for many other microbiological tests. According to ISO 6887-1:2017, BPW is used for the preparation of test samples, initial suspension and decimal dilutions of samples for microbiological examination. Examinations such as colony count (ISO 4833-1:2013) and *Enterobacteriaceae* count (ISO 21528-1:2017) both use BPW as a diluent. In both cases they take a small sub sample of the original sample to carry out the test. Thus, a single food sample can be homogenised via stomaching in BPW to carry out multiple tests. After the samples are taken for the monitoring (colony counts) tests, the same sample can be incubated and used for *Salmonella* testing. Thus, due to its widespread use in testing laboratories it is very difficult to implement alternative media for the enrichment of *Salmonella*. The common adoption of ISO 6579 for *Salmonella* detection means that any new method must provide equivalent performance to this dual enrichment protocol. Alternative methods that reduce the time to result are attractive since the traditional ISO 6579 method takes at least four days. However, single enrichment methods must ensure the target organism has reached detectable levels within a much shorter time frame. To do this an alternative enrichment system needs to prevent the overgrowth of competitive flora by applying selective pressure. This further complicates matters of *Salmonella* detection as low level, stressed target organisms will also be affected by the selective pressure, increasing the risk that they will not reach adequate cell numbers to be detected. Thus, any alternative method must compromise between its ability to promote growth of the target and prevent

overgrowth of non-target organisms. The ISO 6579 standard separates these two stages with BPW providing an environment to resuscitate and multiply, followed by further growth in a selective system with Rappaport-Vassiliadis soya peptone broth (RVS) and Muller-Kauffmann tetrathionate novobiocin broth (MKTTn). A key factor to consider is cost when designing an alternative method, as the extra cost of the method cannot outweigh the time advantages. Alternative methods are inevitably more expensive because the end-point test, such as chromogenic, antibody or genetic detection methods are all more expensive than traditional agars like xylose lysine deoxycholate agar (XLD).

A popular commercial format for an alternative method to ISO 6579 is described as a one broth, one plate (OBOP) method. Even though it is still a traditional cultural method, OBOP methods are described as rapid methods since they offer significant time advantages over the standard methods. An OBOP method is where a single enrichment broth is used, followed by subculture onto a single plate. The benefits compared to ISO 6579 are a reduced time to result and fewer culture media requirements.

Table 4.1 Alternative culture media methods accredited by Association Française de Normalisation (AFNOR) published on October 13th, 2017 available at https://nf-validation.afnor.org/en/food-industry/salmonella-spp/.

Name	Company	Detection Type	Enrichment Media	Enrichment Time	Enrichment Temperature
IBISA	bioMérieux	Chromogenic	BPW + Supplement	16-20 hours	41.5 °C ± 1 °C
IRIS Salmonella	SOLABIA S.A.S	Chromogenic	BPW + Supplement	16-24 hours	41.5 °C ± 1 °C
RAPID' *Salmonella*	BIO-RAD	Chromogenic	BPW + Supplement	16-20 hours	41.5 °C ± 1 °C
SALMA One Day	bioMérieux	Chromogenic	BPW + Supplement	16-24 hours	41.5 °C ± 1 °C
Salmonella PRECIS	Oxoid Ltd	Chromogenic	Proprietary broth	16-20 hours	42 °C
SESAME Salmonella Test	SOLABIA S.A.S	Motility + Chromogenic	BPW	16-20 hours	37 °C ± 1 °C
Simple Method for Salmonella	bioMérieux	Motility	BPW	16-20 hours	37 °C ± 1 °C

Table 4.1 shows the current methods validated against ISO 6579 by AFNOR that utilise culture media (agar) as the end point test. There are further accredited methods that utilise molecular or immunological detection protocols. Most alternative methods use BPW as a base to which a selective supplement is added. It is done in this manner so that the supplement can be added after all routine tests are carried out. If BPW is not used as a base the laboratory must do extra processing which is inefficient and costly. Elevating the temperature of incubation to 41.5 °C ± 1 °C can increase selectivity. The standard incubation temperature for BPW is 37 °C which allows growth of a wide range of microorganisms. The elevated temperature provides greater selectivity as it is less favourable to many non-target organisms, whilst *Salmonella* can still grow well at the higher temperature.

Chromogenic agar for *Salmonella* esterase (CASE) is a diagnostic, selective agar plate for the detection of *Salmonella* and is described in Chapter 3. CASE demonstrated its viability as a chromogenic plating media using the ISO dual enrichment method (Chapter 3, 3.3.11). A single enrichment protocol however, would be beneficial as *Salmonella* contamination could be detected up to one day earlier compared to the protocol described in ISO 6579. To further differentiate any new methodology from what is currently available there should be an improvement on the method. A possible way to improve the efficiency of the proposed method is pooling of samples. Pooling is common place in many pathogen tests where the expectation of negative (absence) results is high. If it can be demonstrated that pooling does not reduce sensitivity, it allows the user to combine several single enrichments into a single test and save time and consumables. Only when a positive result is recorded does the user return to the original single enrichments to discover which one/s contain the target organism. Pooling is common for molecular methods where the detection threshold is relatively low. It is not as common for cultural methods since generally a greater level of target organism is required to yield a positive result, compared to molecular methods.

An enrichment broth and supplement were required to test the suitability of CASE as a rapid method. A supplement was made available from Neogen (USA), prepared by Dr Jerry Tolan. The supplement was designated CNSX and was prepared as freeze-dried vials, which were rehydrated with 10 ml of sterile water. One millilitre of this solution could be added to a 225 ml of BPW prior to incubation with a test matrix. When added as described, the supplement gave the BPW a final formulation of 3 mg/l cefsulodin, 20 mg/l novobiocin, 80 mg/l sulfadiazine and 1.25 g/l xylose in addition to the BPW formulation. Novobiocin is present to suppress or inhibit the growth of many *Enterobacteriaceae,* such as *Klebsiella* spp. and *Proteus* spp. Sulfadiazine suppresses or inhibits many other common background organisms such as *E. coli* which would otherwise compete with *Salmonella* during enrichment. Cefsulodin has specific activity against *Pseudomonas* spp. All these selective agents were chosen as they exert a selective pressure against many of the commonly found background organisms in foodstuffs. Furthermore *Salmonella* spp. are resistant to this selective pressure, meaning they are

afforded a competitive advantage during enrichment. The xylose included is present to help mitigate the strength of the selective pressure on weaker/slow growing *Salmonella* spp., as almost all species can utilise it as a carbon source. The supplement also included a food dye, Fast Green FCF at a final concentration of 2 mg/l. This dye served no biological purpose but was present as a visual indicator of its addition to a test volume, as it turned the BPW bright green.

BPW as described by ISO 6579-1:2017 contains peptone at 10 g/l, sodium chloride at 5 g/l, disodium hydrogen phosphate (anhydrous) at 3.57 g/l and potassium dihydrogen phosphate at 1.5 g/l. This simple media contains a phosphate buffer and sodium chloride to maintain a favourable pH and osmotic balance, respectively, during enrichment. The peptone constituent is to supply all the required carbon and nitrogen required for bacterial growth. Peptone is a vague description thus could be made up of various peptone sources (casein, meat, soya etc). It is also common for media manufacturers to supplement peptone with various extracts to improve the availability of micronutrients, such as vitamins. The CNSX supplement was originally designed for use with a nutrient rich BPW formulation also designed at Neogen by Dr Jerry Tolan. This formulation was designated aBPW and is a deviation of the ISO formulation as its peptone composition is 7 g/l yeast extract, 1.5 g/l soya peptone and 1.5 g/l casein peptone. The yeast extract in the formulation helps mitigate the relatively high levels of selectivity employed in the CNSX supplement. The CNSX supplement and the aBPW were originally designed to be paired with the Neogen genetic detection platform, ANSR. The formulation and protocol had not been paired with any agar medium and was unsuccessful in completing its third-party validation trials.

Using the CNSX selective supplement and aBPW provided as a starting point, the main aim of the work was to establish an enrichment method and protocol that would allow CASE to be utilised in an OBOP methodology, with all common foodstuffs. It is critical that real matrices are used since it has been shown that pure culture experiments fail to replicate the issues of competitive growth dynamics, inhibitory effects and dilution issues experienced by testing laboratories (López-Campos *et al*., 2012).

Furthermore, it is necessary to test any one broth strategy with high background flora from natural contaminated source, as it has been demonstrated this to be a major problem for enrichment methods (Margot *et al*., 2015).

The key questions addressed were:

- What is the optimal BPW and selective supplement formulation to achieve a single step enrichment protocol, for *Salmonella enterica* from foodstuffs?
- What is optimal subculture volume for the methodology?
- Can pooling up to 5 samples be achieved without a reduction in sensitivity?
- What are the effects of different matrices?
- What is the limit of detection for *Salmonella* species for an OBOP methodology?

4.2 Methods

All formulations of BPW and selective supplements described, are detailed in Appendix 2.

Figure 4.1 displays the simplified protocol for the single stage enrichment procedures described in this chapter. Deviations from this protocol are described in each methodology section.

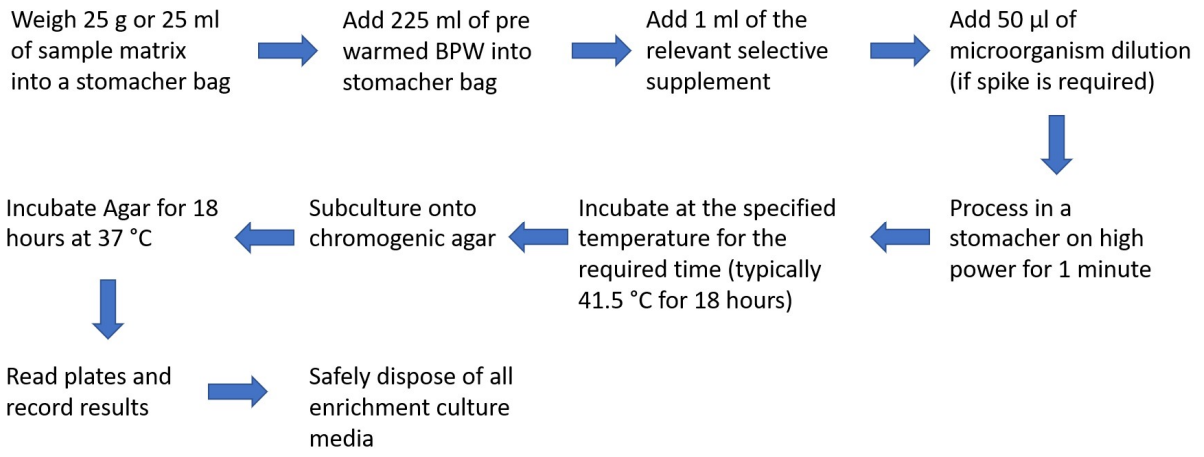


Figure 4.1 Basic protocol of the single stage enrichment procedures described in chapter 2.

4.2.1 Subculture experiment

Naturally colonised chicken wings were sourced from a local supermarket retailer. Skin was harvested from the wings by using a sterile scalpel and collected in sterile containers. The custom version of BPW (aBPW) made by Neogen, designed to pair with the selective supplement, CNSX was prepared to the manufacturer instructions. 25 g of chicken skin was added to a stomacher bag followed by 225 ml of prepared aBPW + the CNSX supplement, pre-warmed to 41.5 °C. Each bag was then inoculated with 50 µl of a target dilution of overnight enriched culture. The same volume of the dilution was spread onto a TSA plate (LAB011, Lab M), and incubated at 37 °C for 18 hours for enumeration. *Salmonella* Enteritidis ATCC 13076 (Sale76) was used as a target organism and *Enterobacter aerogenes* ATCC 13048 (Entba48) was used as a non-target organism. The combinations stated in Table 4.2 were used with the described designations.

Table 4.2 Combination of test material used for the subculture experimentation.

(1)	Raw Chicken Skin + Sale76 + Selective Supplement
(2)	Raw Chicken Skin + Sale76
(3)	Raw Chicken Skin + Sale76 + Entba48 + Selective Supplement
(4)	Raw Chicken Skin + Sale76 + Entba48

The target organism was inoculated at a low level (10-50 CFU) and the non-target organism was inoculated at a high level (approximately 10^3-10^4 CFU). Samples were homogenised (Stomacher 400, Seward) for 60 seconds on the high setting. All samples were incubated at 41.5 °C for 20 hours. Samples were subcultured onto both versions of the chromogenic agar described in chapter 3 (Table 3.3), since at this point it had not been determined which version was superior. Subculture was performed on the nonanoate and caprylate chromogenic plates at 18 and 20 hours with two different volumes, 10 µl and 50µL. Sterile, disposable, calibrated 10µL loops (TS/2-B, TSC) were used for the 10 µl subculture. The same kinds of loops were used to streak 50 µl deposited by pipette for the 50 µl

subculture. The chromogenic plates were then incubated at 37 °C for 18-20 hours. All tests were duplicated.

4.2.2 Pooling experiment

The exact same protocol of preparation was followed as the previously described subculture experiment (4.2.1), using naturally contaminated chicken skin as a matrix. The combinations stated in Table 4.3 were used with the stated numerical designations.

Table 4.3 Combination of test material used for the pooling experiment.

1	Raw Chicken Skin + Selective Supplement + Sale76
2	Raw Chicken Skin + Selective Supplement +Sale76 + Entba48
3	Raw Chicken + Selective Supplement

After 18 hours enrichment at 41.5 °C, aliquots of each sample were pooled into a sterile plastic universal tube to create four separate pooled samples. The pooled samples were made up as described in Table 4.4. The combinations were chosen to emulate various levels of contamination possibly encountered in naturally colonies food matrices.

Table 4.4 Combinations of samples described in Table 4.3 to make up pooled test samples.

	1	**2**	**3**
Pool 1	1mL		4mL
Pool 2		1mL	4mL
Pool 3	1mL	1mL	3mL
Pool 4	2mL		3mL

The pooled samples were then subcultured in duplicate onto both the nonanoate and caprylate chromogenic plates (as described in Table 3.3) at 10 µl, 20 µl, 30 µl, 40 µl & 50 µl volumes, dispensed by pipette and streaked with sterile loops. The individual samples (1, 2 and 3) were also subcultured (10 µl) onto the chromogenic agars directly. All agars were incubated for 18 hours at 37 °C.

4.2.3 Temperature experiment

21 *Salmonella* species pure cultures, 2 mixed cultures and 9 non-target organisms (all designated in Table 3.1 & 3.2), were separately inoculated into 10 ml aliquots of BPW. In all cases the *Salmonella* cultures were dosed at 10-100 CFU, whilst all non-target organisms were dosed at ~10^4 CFU. The custom aBPW used in the subculture experiment was compared against Lab M ISO BPW (LAB204). Both media were supplemented with CNSX selective supplement. All tests were duplicated and incubated at 37 °C and 41.5 °C, followed by subculture at 16 h, 18 h and 24 h. Subculture was performed onto CASE, which was incubated for 18-24 hours at 37 °C and examined for typical colonies. Subculture was performed with a 10 µl loop streaked onto the surface of the agar into four quadrants. Pure culture *Salmonella* isolates were only subcultured at 16 h. All other tests were subcultured at 16 h, 18 h and 24 h.

4.2.4 BPW sensitivity experiment

The exact same protocol of preparation was followed as the previously described subculture experiment (4.2.1), using naturally contaminated chicken skin as a matrix. Two different Lab M BPW formulations, LAB046 and LAB204 were compared with the CNSX supplement. These two formulations differ from the previously mentioned aBPW formulation, by their peptone composition. All tests used 25 g of raw chicken skin with 225 ml of BPW, which were spiked with low levels of *Salmonella* and/or competitive non-target organisms. The combinations stated in Table 4.5 were used with the stated numerical designations.

Table 4.5 Inoculation schedule for the BPW sensitivity experiment. Short code designations available in Table 3.2.

1	+ Sale76 (10-100 CFU)
2	+ Sale76 (1-10CFU)
3	+ Salv42 (10-100CFU)
4	+ Salv42 (1-10CFU)
5	+ Sale76 (10-100CFU) + Entba48
6	+ Sale76 (1-10CFU) + Entba48
7	+ Salv42 (10-100CFU) + Entba48
8	+ Salv42 (1-10CFU) + Entba48
9	+ Sale76 (10-100CFU) + Ec22
10	+ Sale76 (1-10CFU) + Ec22
11	+ Salv42 (10-100CFU) + Ec22
12	+ Salv42 (1-10CFU) + Ec22

The target organisms were inoculated at the stated levels and the non-target organisms were inoculated at a high level (approximately 10^3-10^4 CFU). All samples were incubated at 41.5 °C for 18 hours. Samples were subcultured (10 µl) onto CASE and incubated for 18 hours at 37 °C. The *Salmonella* chosen as the targets were selected due to their sensitivity to the selective agents employed. *S.* Typhimurium ATCC 14028 is often used as a marker organism for culture media formulation but previous experience has shown it to be much more resistant to selective pressure than the *S.* Enteritidis and *S.* Virchow. All tests were performed in duplicate.

4.2.5 New supplement experiment

A revised supplement formulation was prepared in collaboration with Dr Jerry Tolan at Neogen. The concept was that a less selective supplement could be paired with a less nutritious BPW, to improve the growth of *Salmonella* without permitting over growth of non-target. The new supplement omitted the cefsulodin and now contained 25 mg/l novobiocin, 40 mg/l sodium sulfadiazine and 0.5 g/l D-mannitol. The omission of the cefsulodin and reduction of the sulfadiazine was to improve the recovery of the target organism, by reducing the negative impact on *Salmonella*. Since CASE already contains cefsulodin to inhibit *Pseudomonas* species it was not completely necessary to have it present

during enrichment. The increase in novobiocin was to improve the inhibition of non-target. This increase did not affect *Salmonella* since the other two selective agents had either been removed or reduced. The new supplement was designated NSM and was made by dissolving all components in dH_2O, filter sterilising through a 0.2 µm membrane filter and adding to the BPW after sterilisation at the previously mentioned concentrations. After filter sterilisation, the supplement could be stored at 2-8 °C for up to a week without any loss in activity. Fast green was not incorporated into the supplement, but extra care was taken to ensure the supplement was added when required. The NSM supplement was prepared and tested in three different BPW formulations. All contained the same ISO level of phosphate buffer and sodium chloride but had different nutritional components. C3M3S3V1 contained 3 g/l tryptone, 3 g/l meat peptone, 3 g/l soya peptone and 1 g/l vitamin mix (previously described in 3.3.3). LAB204 contained only 10 g/l tryptone, and LAB046 contains 5 g/l tryptone and 5 g/l meat peptone. All formulations were prepared in the same manner and sterilised at 121 °C for 15 minutes. Raw, unpasteurised milk was used as the test matrix as it introduces a relatively large amount of protein and carbohydrates, which can shift the available nutrients during enrichment from that which is present in the BPW. This would help identify if the selectivity implemented was too highly influenced by the test matrix. The raw milk was naturally contaminated and sourced from a local dairy farm bulk milk tank. All tests were carried out as 25 ml of raw milk in 225 ml of prepared BPW in a stomacher bag. Target *Salmonella* were inoculated at 1-10 CFU and *Pseudomonas* was inoculated at a high level (approximately 10^3-10^4 CFU). All organisms were inoculated as a 50 µl addition to the BPW. All organisms were enumerated on TSA.

The combinations as described in Table 4.6 were prepared in triplicate with the stated numerical designations.

Table 4.6 Combination of test material utilised in the new supplement experiment. Organism short codes specified in Table 3.2.

Key		1	2	3	4
A	C3M3S3V1	+Milk	+ Milk +Sale76	+ Milk +Salv77	+ Milk +Sale76 + Psa53
B	LAB204	+Milk	+ Milk +Sale76	+ Milk +Salv77	+ Milk +Sale76 + Psa53
C	LAB046	+Milk	+ Milk +Sale76	+ Milk +Salv77	+ Milk +Sale76 + Psa53

All tests were incubated for 18 hours at 41.5 °C and subcultured (10 µl) on CASE, which was further incubated for 18 hours at 37 °C. Tests were scored as positive if one or more typical colonies were identified by latex test on CASE.

4.2.6 Matrices experiment

Five matrix materials were selected to challenge the new supplement formulation paired with the LAB204 BPW. Raw (unpasteurised) milk, raw chicken skin, unpasteurised soft cheese (Brie), raw high fat (20 %) pork mince and unwashed organic salad leaves were selected as the challenge material. The milk, as previously described, challenges the method due to the input of protein and carbohydrate which changes the nutritional properties of enrichment. The soft cheese normally has a high bio burden and causes difficulties in subculture, as the broth is usually not homogenous due to the texture and fat content. Also, like the milk it adds more protein and carbohydrates. The high fat mince can prevent the sample being homogenous at subculture due to the fat content causing entrapment of microorganisms in globules, non-uniformly. The chicken skin typically introduces a high level of competitive microorganisms. The organic, unwashed salad leaves are difficult to properly homogenise in BPW and are likely to have a high bio burden since they have not been treated, physically or chemically, to remove contamination. All matrices except the unpasteurised soft cheese and milk were purchased from a supermarket retailer. The unpasteurised products were not readily available in the UK. The milk was again sourced from a local dairy farm and the unpasteurised Brie was purchased from a UK distributor of French sourced cheese.

To mimic natural contamination, the matrix samples were prepared in 25 g amounts and inoculated with 50 µl of low level (1-10 CFU) *Salmonella* Enteritidis ATCC 13076. These individual samples were then stored at 2-8 °C for 72 hours prior to being added to BPW for enrichment. This was to simulate cell stress experienced by microbial contamination of chilled foodstuffs. Matrix material (25 g) was stomached for 60 seconds on high with 225 ml of BPW + the new supplement formulation. Three versions of BPW were tested with the new supplement formulation (NSM) as described in the new supplement experiment. LAB204 was tested against C4M5V1 and C5Y5. All contained the same ISO level of phosphate buffer and sodium chloride but had different nutritional components. C4M5V1 contained 4 g/l tryptone, 5 g/l meat peptone and 1 g/l vitamin mix. C5Y5 contained 5 g/l tryptone and 5 g/l yeast extract. LAB204 is as previously described.

Each spiked matrix was replicated 10 times. After 18 hours incubation at 41.5 °C, 10 µl of each enrichment was subcultured onto XLD and CASE. These plates were incubated for 18 hours at 37 °C and examined for typical colonies. 1 ml of each of the original enrichments was also added to 9 ml of RVS and incubated for 24 hours at 41.5 °C. After this secondary enrichment, all samples were subcultured (10 µl) onto both CASE and XLD and incubated, for 18 hours at 37 °C and examined for typical colonies. Up to three typical colonies were picked for confirmation by a *Salmonella* latex agglutination test (Oxoid).

To assess the level of natural contamination, 25 g each matrix was homogenised by stomaching for 60 seconds on high power in a separate aliquot of 225 ml of BPW (LAB204). Several serial dilutions were performed in MRD from the homogenised samples and these were spiral plated onto TSA plates. The TSA plates were incubated at 37 °C for 18 hours. The plates with colonies between 0-250 CFU were counted. From these counts the CFU/g was calculated for each matrix sample.

4.2.7 Alternative method vs. ISO with challenging matrices experiment

The exact same protocol was followed as described in the matrix experiment (4.2.6), but just using LAB204 as the BPW and NSM supplement. The OBOP method was compared against a modified ISO

6579 workflow. The modification was that MKTTn was omitted and only RVS was utilised as the secondary enrichment. The OBOP plate method used LAB204 plus the NSM supplement incubated at 41.5 °C for 18 hours and subcultured (10 µl) onto CASE. The ISO method used LAB204 incubated at 37 °C for 18 hours, followed by a 1 ml subculture into 9 ml of prepared RVS, incubated at 41.5 °C for 24 hours. This was then subcultured (10 µl) separately onto CASE and XLD. All agar plates were incubated for 18 hours at 37 °C and examined for typical colonies. In all cases, matrices were spiked and stress by holding at 2-8 °C as described in the previous matrix experiment (4.2.6) and homogenised in a stomacher for 60 seconds on high power. Raw (unpasteurised) milk, raw chicken skin, and unpasteurised soft cheese (Brie) were used as matrix material. All matrices were spiked with a low level (~10 CFU) of *Salmonella* Enteritidis ATCC 1307, except for the Brie which was also spiked with a very low (~5 CFU) level. These matrices were selected as they proved the most difficult in the previous experiment. The Brie was tested with a lower inoculum as it had the greatest impact on recovery in the previous matrix experiment. All samples were replicated 10 times.

4.3 Results

4.3.1 Subculture experiment

The aim of the subculture experiment was to assess if there was any benefit in a larger (50 µl) subculture volume compared to the standard 10 µl subculture volume. Enumeration counts on TSA showed that *S.* Enteritidis (Sale76) was inoculated at 16 CFU and *E. aerogenes* (Entba48) was inoculated at approximately 18,000 CFU.

Table 4.7 Detection results for the subculture experiment (4.2.1), at both time points with 50 µl and 10 µl subculture volumes. += positive detection of *Salmonella*, (w) =poor intensity/small colony, -=No *Salmonella* detected. See Table 4.2 for sample key. Individual results separated by / for duplicates.

18 Hours	50 µl Subculture		10 µl Subculture	
Sample	Nonanoate	Caprylate	Nonanoate	Caprylate
(1)	+/+	(w)+/+	+/+	+/+
(2)	+/-	(w)+/-	+/+	(w)+/-
(3)	+/+	+/+	+/+	+/+
(4)	-/-	-/-	-/-	-/-
20 Hours	50 µl Subculture		10 µl Subculture	
Sample	Nonanoate	Caprylate	Nonanoate	Caprylate
(1)	(w) +/+	(w) +/+	+/+	(w) +/+
(2)	(w) +/-	(w)+/+	(w) +/+	(w)+/+
(3)	+/+	+/+	+/+	+/+
(4)	-/-	-/-	-/-	-/-

There appeared to be no benefit in using a larger subculture volume. In fact, in most cases positives were either harder to see due to overcrowding on the agar by non-target organisms or were undetectable. The effect of overcrowding is displayed in Figure 4.2.

Figure 4.2 Appearance of the same samples subcultured with 50 µl (left) compared with 10 µl (right) on to CASE. Blue/green colonies are *Salmonella*, black colonies are *E. aerogenes.*

The 50 µl subculture compared to the 10 µl subculture consistently yielded more black β-glucosidase positive colonies making the green *Salmonella* colonies harder to pick out. Table 4.7 shows that in most cases *Salmonella* was identified from both subculture volumes except for when the selective supplement was not included. Even though *Salmonella* was detected on most of the tests, 10 µl produced more single target colonies as shown in Figure 4.2.

The 18-hour subculture performed slightly better than the later 20-hour subculture. There were more positive results at the 20-hour subculture that were classed as weak because there was an increase in non-target colonies, when compared to the 18-hour subculture. After this experiment it was decided that a 10 µl subculture volume and an 18-hour enrichment time was optimum for *Salmonella* detection with the proposed one broth enrichment protocol.

4.3.2 Pooling experiment

The pooling experiment examined the possibility of pooling individual enrichment samples so that less culture media and time would be consumed when testing multiple samples. Table 4.8 shows the results of the experiment detailed in 4.2.2.

Table 4.8 Detection results for all pooled samples for the pooling experiment (4.2.2) at various subculture volumes on both versions of the chromogenic plates. +=positive, (w) =weak/small colonies. Individual results for duplicate samples are separated by /. See Table 4.4 for pool description.

Pool 1	10 µl	20 µl	30 µl	40 µl	50 µl
Nonanoate	+/+	+/+	+/+	+/+	+/+
Caprylate	(w)+/(w)+	+/+	+/+	(w)+/(w)+	(w)+/+

Pool 2	10 µl	20 µl	30 µl	40 µl	50 µl
Nonanoate	+/+	+/+	+/(w)+	+/+	+/(w)+
Caprylate	(w)+/(w)+	+/+	+/+	(w)+/(w)+	(w)+/(w)+

Pool 3	10 µl	20 µl	30 µl	40 µl	50 µl
Nonanoate	+/+	+/+	+/+	+/+	+/(w)+
Caprylate	(w)+/+	+/+	+/+	(w)+/(w)+	(w)+/(w)+

Pool 4	10 µl	20 µl	30 µl	50 µl
Nonanoate	+/+	+/+	+/+	+/(w)+
Caprylate	+/+	+/+	(w) +/+	(w)+/(w)+

Direct (10 µl) subculture of sample combination 1 & 2 but not 3 (defined in Table 4.3), without pooling yielded positive recovery and detection of *Salmonella* on both versions of the chromogenic plates. This showed that the single sample methodology could detect Salmonella from these samples. Sample 3 had not been artificially inoculated with *Salmonella* and when tested, did not yield a positive result. This was a control of the experiment to test if the matrix material was already naturally contaminated with *Salmonella*, which it was not. Figure 4.3 displays what typical positive results looked like from the pooled samples. Note how *Salmonella* (blue/green colonies) are grossly in the minority of the colonies growing on the plates.

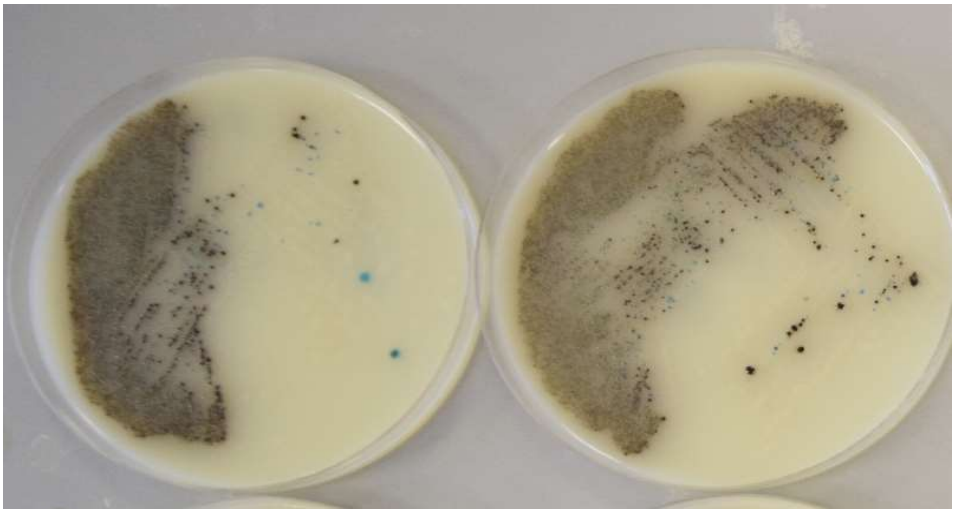


Figure 4.3 Subculture of Pool 1 (as described in 4.2.2) on CASE with 10 µl (left) and 20 µl (right), both yielding positive results. Blue/green colonies are *Salmonella*, black colonies are non-target competitive microorganisms.

4.3.3 Temperature experiment

The temperature experiment tested two different formulations of BPW against each other at both the standard temperature of 37 °C and the elevated temperature of 41.5 °C. All tests utilised the CNSX described in 4.1. A full table of results is available in Appendix 3.

The aBPW formulation produced higher qualitative recovery of both target and non-target organisms at both temperatures, compared to the standard ISO BPW (LAB204). The standard ISO BPW yielded fewer colonies for most of the pure culture *Salmonella* enrichments but did achieve positive recovery of all *Salmonella* cultures tested at both temperatures. The ISO BPW also resulted in generally superior inhibition of the non-target microorganisms. There was not a major difference in either BPW formulations regarding the recovery of *Salmonella* at either temperature. However, there was a clear difference regarding selectivity, with 41.5 °C achieving much greater suppression of non-target microorganisms compared to 37 °C. At 37 °C aBPW failed to detect *Salmonella* in both the mixed culture inoculations, most likely due to the overgrowth of the non-target organisms. At the lower temperature *Pseudomonas* was not inhibited, resulting in false positives on the agar. The cefsulodin

incorporated in the CNSX supplement appears not to be effective in liquid culture at 37 °C, in this formulation but was at 41 °C. *E. coli* ATCC 8739 was much more suppressed at 37 °C compared to 41 °C in the aBPW. The LAB204 BPW did not suffer any selectivity issues at either temperature but did generally yield lower recoveries of *Salmonella*. LAB204 contains only tryptone to promote growth and as such, results in lower titres of all organisms compared to aBPW. At 41.5 °C some *Salmonella* were recovered in low numbers, but there was excellent suppression and/or inhibition of the non-target organisms.

The 18 and 20 hour subcultures of the mixed culture tests did not improve on the result of the first (16 hour) subculture. As the enrichment time increased, more non-target colonies were present on the agar. The same pattern of increased growth was observed for the non-target pure culture tests, with the prolonged enrichment time subcultures.

4.3.4 BPW sensitivity experiment

The BPW sensitivity experiment tested the performance of two different versions of BPW against each other, both using the CNSX supplement described in 4.1. The samples used (described in Table 4.5) represented a highly challenging test sample that may be encountered during any potential method validation protocol. Table 4.9 displays the results of the challenge test, Table 4.10 displays the enumeration counts for the artificial inoculums used.

Table 4.9 Results of the subculture on CASE from all inoculated samples from the BPW sensitivity experiment (4.2.4). +=recovery and detection of *Salmonella*, -= no *Salmonella*_detected, (w) =weak/low number recovery. Individual results for duplicate samples are separated by /. See Table 4.5 for sample key.

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
LAB046	+/+	+/+	+/+	+/-	+/+	+/-	+/ +	+	(w) +/-	(w) + / -	+/ +	+/ +
LAB204	- / +	- / -	+ / -	- / -	(w) +	(w) +	+	+	(w) +	-	-	-

Table 4.10 Enumeration of 50 µL of inoculums used in the BPW sensitivity experiment (4.2.4). Short code designations available in Table 3.2.

	Target	Inoculum level expressed as CFU
Sale76	10-100 CFU	58
	1-10 CFU	5
Salv42	10-100 CFU	99
	1-10 CFU	8
Entba48	10^3-10^4 CFU	23211
Ec22	10^3-10^4 CFU	42087

Overall LAB046 outperformed LAB204 in its ability to recovery *Salmonella*. Based on these results, LAB046 + CNSX supplement was trialled at a third-party site against the ISO 6579 workflow and failed to match the sensitivity of the ISO 6579 control method (data not shown).

4.3.5 New supplement experiment

The BPW sensitivity experiment showed that LAB204 + CSNX supplement was too selective. Thus, a new supplement was designed that was less selective. The aim was to pair a less selective supplement formulation with a less nutritionally rich BPW, to achieve optimal performance with mixed culture enrichment. Enumeration of the spiked cultures showed that *S.* Enteritidis (Sale76) was inoculated at 3 CFU, *S.* Virchow (Salv42) at 10 CFU and *P. aeruginosa* (Psa53) at approximately 12,000 CFU. The results of the subculture are displayed in Table 4.11.

Table 4.11 Results of the subculture on CASE from all inoculated samples from the new supplement experiment (4.2.5). +=recovery and detection of *Salmonella*, -= no *Salmonella* detected. See table 4.6 for key.

Results		1	2	3	4
A	C3M3S3V1	-/-/-	-/-/-	+/-/+	-/+/-
B	LAB204	-/-/-	+/+/+	+/+/+	+/+/+
C	LAB046	-/-/-	-/-/+	+/+/-	-/+/-

LAB204 was superior to the other two BPW formulations, successfully recovering *Salmonella* in all spiked tests. Test 1 did not yield any positive results with any enrichment, meaning that no naturally contaminated *Salmonella* could be detected. Since cefsulodin had been removed from the selective supplement, *Pseudomonas* was spiked at a high level in test 4 to see if false positives from the organisms were seen on the agar. The test organism, Psa53 presents bright blue colonies when present in high enough numbers on CASE. Thus, it was possible to differentiate any from blue/green *Salmonella* colonies. None of the BPW formulations yielded any false positives with *Pseudomonas.* This demonstrated that the cefsulodin present in CASE was adequate to inhibit any *Pseudomonas* present.

4.3.6 Matrices experiment

The matrices experiment tested three versions of BPW + NSM supplement (described in 4.2.6) against each other using challenging matrices material, and a low-level stressed (~10 CFU, stressed by holding at 2-8 °C) spike of *S.* Enteritidis. Subculture for both CASE and XLD were performed from the same enrichment. A full table of results is available in Appendix 4. Enumeration of the artificially spiked *Salmonella* showed that *S.* Enteritidis was inoculated at 8 CFU. Total viable (aerobic) count (TVC) analysis of each matrix type was carried out on each sample without enrichment or artificial spiking.

This analysis showed that unpasteurised milk contained 3.78 x 10^4 cells/25 ml, chicken skin contained 4.32 x 10^5 cells/25 g, unpasteurised soft cheese contained 3.6 x 10^6 cells/ 25 g, pork mince contained 4.5 x 10^2 cells/25 g and organic salad leaves contained 6.03 x 10^6 cells/25 g.

The matrix test was more challenging than the previously described experiments. By spiking matrix with a low level of *Salmonella* and then holding in the fridge for 72 hours, this test better mimicked what would be carried out at third party validation sites. Two more custom formulations of BPW were tested against LAB204 (all with the improved NSM supplement), but neither were superior to LAB204. This experiment also implemented a secondary enrichment step in RVS and dual plating onto XLD as well as CASE. By doing this it can be assumed that if the single enrichment step subcultured onto CASE yields the same result as the plating after secondary enrichment, the method is comparable to the standard ISO 6579 method. Also, if the single enrichment step plating result is the same as it is after RVS secondary enrichment, *Salmonella* is being outcompeted in the original enrichment. The RVS secondary step allows for further multiplication, so it is expected that some originally negative results may become positive after RVS enrichment. However, Figure 4.4 shows that RVS also gave competitive organisms opportunity to grow further, resulting in more non-target colonies on the agar. The C4M5V1 BPW recorded poor results with the chicken skin matrix, but LAB204 and C5Y5 BPW performed better. The higher peptide availability in C4M5V1 seems to be detrimental to the suppression of competitive Gram-negative organisms, resulting in a high incidence of detection failure. The most challenging matrix was the unpasteurised cheese, with all three BPW formulations performing poorly. It is worth noting that the traditional workflow (RVS onto XLD or CASE) also performed poorly, suggesting that the spiked *Salmonella* were not adequately maintained during the 72-hour holding time. XLD generally performed worse than CASE due to the high number of false positives that were present, increasing the difficultly of successfully confirming *Salmonella*. It was interesting to note that even though the salad leaves had the highest bioburden, they did not present the greatest challenge for competitive enrichment. This suggests that it is the specific competitive

organisms present, not necessarily the number, which poses the greatest issue in recovering *Salmonella* in a mixed enrichment culture.

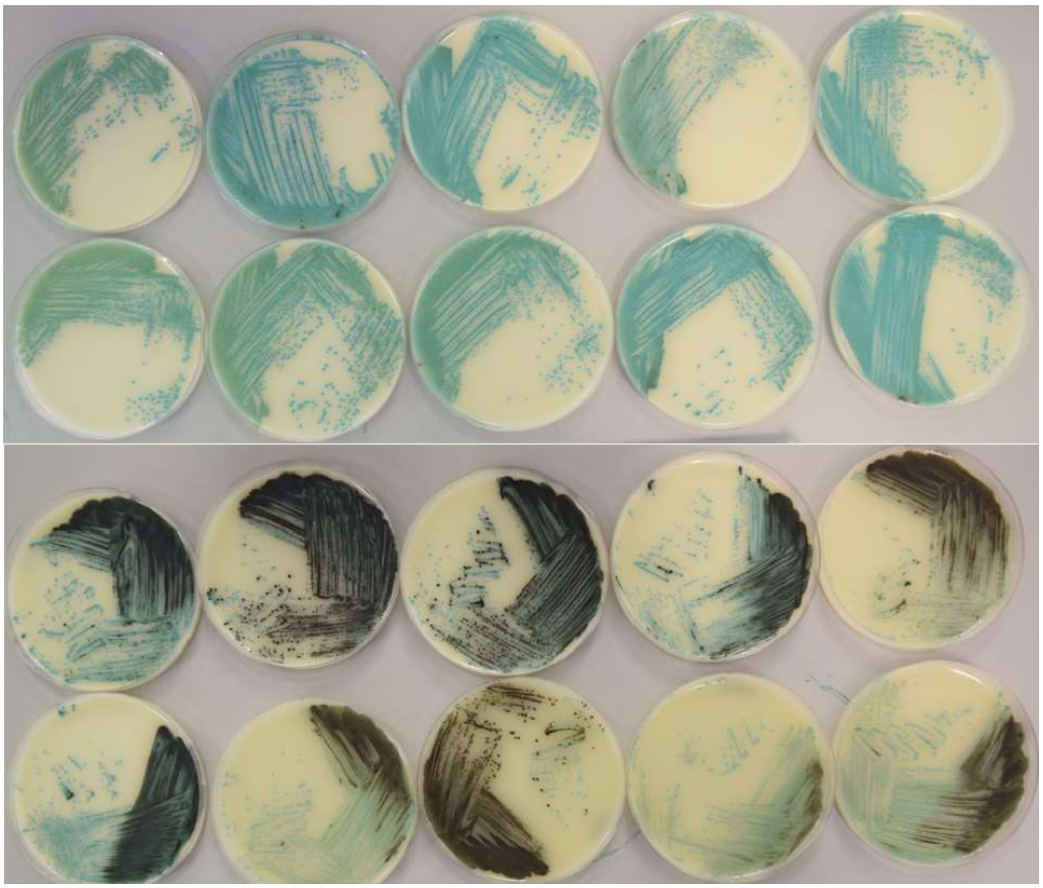


Figure 4.4 Pork mince samples subcultured on CASE from LAB204 (as described in 4.2.6), directly from primary enrichment (above) and after RVS secondary enrichment (below). Blue/green colonies are *Salmonella*, black colonies are non-target competitive microorganisms.

4.3.7 Alternative method vs. ISO with challenging matrices experiment

The final matrix challenge compared the performance of the proposed one broth methodology against the ISO 6579 method. The ISO BPW (LAB204) was used for both methods, except that the media was combined with the NSM selective supplement for the one broth methodology. Enumeration of the artificially spiked *Salmonella* showed that *S.* Enteritidis (Sale76) was inoculated at 9 CFU for the low level (~10 CFU) challenge and 2 CFU for the very low challenge (~5 CFU).

Table 4.12 Results of spiked matrix testing with OBOP and ISO methodologies (as described in 4.2.7).

Refer to table key for result codes

Table Key	
m+?	Positive, minority of colonies, small/weak colonies
m+	Positive, minority of colonies
M+	Positive, majority of colonies
NEG	Negative

Matrices	Replicate Number	One Broth	ISO 6579/RVS Subculture	
		CASE	XLD	CASE
Raw (unpasteurised) Milk + ~10 CFU Sale76	1	M+	M+	M+
	2	M+	M+	M+
	3	M+	m+	M+
	4	M+	m+	M+
	5	M+	m+	M+
	6	m+	m+	m+
	7	m+	m+	m+
	8	m+	m+	m+
	9	NEG	m+	m+
	10	NEG	NEG	m+
Chicken skin + ~10 CFU Sale76	1	M+	m+	M+
	2	M+	m+	M+
	3	M+	m+	M+
	4	M+	m+	M+
	5	M+	m+	M+
	6	M+	m+	m+
	7	M+	m+	m+
	8	M+	m+	m+
	9	m+	m+	m+
	10	m+	NEG	m+

Matrices	Replicate Number	One Broth	ISO 6579/RVS Subculture	
		CASE	XLD	CASE
Unpasteurised soft French cheese (Brie) + ~10 CFU Sale76	1	M+	m+	M+
	2	M+	m+	M+
	3	M+	m+	M+
	4	M+	m+	M+
	5	m+	m+	M+
	6	m+	m+	m+
	7	m+	m+	m+
	8	m+	NEG	m+
	9	NEG	NEG	NEG
	10	NEG	NEG	NEG
Unpasteurised soft French cheese (Brie) + ~5 CFU Sale76	1	m+	M+	m+
	2	NEG	M+	m+
	3	NEG	M+	m+
	4	NEG	m+	m+
	5	NEG	m+	m+
	6	NEG	m+	m+
	7	NEG	NEG	NEG
	8	NEG	NEG	NEG
	9	NEG	NEG	NEG
	10	NEG	NEG	NEG

Table 4.12 shows the full results from the experiment. In most cases the one broth methodology achieved the same sensitivity as the ISO 6579 methodology. A high level of failure was seen in the very low-level inoculation for the unpasteurised soft cheese. The ISO method detected *Salmonella* in 60 % of the samples, whereas the proposed enrichment only achieved 10 % detection. It's worth noting that when the same matrices when inoculated with greater numbers (~10 CFU) achieved 80 % recovery with both methods. This suggests that the limiting factor is sensitivity in terms of required cell density initially present, in challenging matrices like unpasteurised cheese with the one broth methodology.

4.4 Discussion

From this study, several observations can be made. An increased nutritional composition of BPW was in most cases detrimental to the recovery of *Salmonella*. The high peptide composition of some of the BPW formulations designed favoured the growth of the competitive microorganisms, which were typically present in much higher numbers initially. It has previously been shown that the peptide composition of BPW has a critical effect on *Salmonella* recovery (Gray *et al*., 2008). However, it is difficult to find literature data that describes the effect on growth dynamics of selective supplementation of BPW, in relation to the peptide composition. The optimal composition of any selective supplement for this purpose must complement the base enrichment broth. This is so that extra selectivity and growth promoting agents are not added just to cancel each other out. With other selective pressure from an antibiotic supplement like NSM, the elevated temperature of 41.5 °C is appropriate for single step enrichment. Also, 18 hours is adequate to achieve detectable levels of *Salmonella*. Prolonged enrichment lessens the effect of the suppression achieved from the selective supplementation. For single sample analysis, a 10 µl subculture is optimal to minimise carryover of non-target microorganisms, which would otherwise obscure the plating medium. The ability to use cultural based detection methods, for the reliable detection of *Salmonella* from pooled samples, appears unlikely for all matrices types without intervention by means of sample preparation, such as immunomagnetic separation (IMS). There are several matrices that pose much greater difficultly for *Salmonella* detection, either due to the background microflora present, or the influence on the nutritional composition of the enrichment environment. The limit of detection of *Salmonella* in the proposed OBOP strategy is theoretically between 1-10 CFU for most matrices. However, those matrices that cause difficulty in detection (such as unpasteurised soft cheese) may require higher numbers of *Salmonella* originally present to yield a positive result. The proposed OBOP strategy was successful in many aspects and could pass an alternative methodology validation (ISO 16140), but only for certain matrix types. To possibly achieve comparable results against ISO 6579, further fine tuning would have to be carried out to reach a balance between selectivity and growth promotion. One

aspect not critically examined in this study was the diversity of *Salmonella* spp., regarding their tolerance of the employed selective agents. It is likely that any selective cultural method will bias the chance of detection of certain serovars, based on their susceptibility to selective compounds and the concentrations used. Any alternative method is not only judged on its ability to match the performance of the control, but in what convenience it offers in terms of time and resource. Thus, it is acceptable for an alternative method to have limitations, if they are well described and can be accounted for.

The common subculture volume from enrichment broth to agar plate is 10 µl. This is because in most traditional methods subculture is performed after secondary enrichment, where the target pathogen should have reached sufficient number so that 10 µl contains enough colony forming units to yield a positive result on the agar. With a single step enrichment strategy, the CFU/ml will inevitably be lower due to the shorter total time of enrichment. Therefore, the logic of a larger subculture volume (i.e. 50 µl), is that this will improve the chances of detecting *Salmonella* colonies on the agar. The experimentation showed that whilst the larger volume did indeed carry over more cells, it carried over more non-target organisms that crowd the plate obscuring the target colonies. Thus, for single sample enrichment protocols (like the OBOP methodologies described in 4.1), the common 10 µl subculture onto an agar plate is superior to 50 µl. The results of the subculture and pooling experiments reinforced the findings in Chapter 3, that the nonanoate (CASE) version of the chromogenic plate is superior to the caprylate version. This can largely be attributed to the contrast between the target and non-target colonies being superior in the nonanoate base. When the selective supplement was omitted in one of the test in the subculture experiment, it is likely that the low numbers of spiked *Salmonella* were out competed during the enrichment. The high numbers of non-target organisms are reaching a critical level before the *Salmonella* can reach detectable levels, forcing the *Salmonella* to prematurely enter the stationary phase (i.e. the Jameson effect). Since there is no selective pressure being applied to the non-target organisms and they significantly outnumber the target, overgrowth occurs which causes a test failure. The subculture experiment suggests that in the presence of high

background microorganisms, enrichment more than 18 hours is not beneficial to *Salmonella* detection. This is because the prolonged enrichment encourages the further growth of competitive organisms, resulting in a greater number of colonies on the agar subculture.

The ability to pool up to 5 samples was assessed with a variable degree of success. The experiment was designed so that most of the pooled samples were negative for *Salmonella*, so that the *Salmonella* present in the other samples were diluted. Despite the conclusion of the subculture experiment an increasing range of subculture was performed to mitigate the dilution effect of pooling. For a weak positive result, only a single identifiable colony is required. Pooling samples using the implemented protocol appears to result in success. However, the results were by no means clear and the positive results were weak at best with typically ≤ 2 CFU of *Salmonella* per plate. This level of recovery is too low to be routinely detected by most food testing laboratories, where clarity of results is critical, due to time constraints. This is because any given laboratory could be analysing hundreds of samples each day, if the result is not clear i.e. if *Salmonella* is hard to visually identify, there is a risk of an incorrect result being reported. A pooling protocol must be as sensitive as a single sample protocol to be implemented. If the sensitivity is lower, positive samples that are pooled may be missed. Based on the results of this limited study it was concluded that it was unlikely that a single enrichment strategy based on cultural methods alone, would be sensitive enough to achieve comparable performance to ISO 6579 when pooling up to 5 samples. In many instances, the proposed protocol would most likely perform adequately, but it is unlikely that it would perform for all matrix types and all levels of bacterial contamination. Pooling of samples for *Salmonella* detection is possible, and has been described for non-cultural end point tests, such as immunoassays and nucleic acid based detection methods (Eijkelkamp *et al.*, 2009). Due to a much lower limit of detection compared to agar methods, these alternative methods can detect much lower level of *Salmonella* per ml of enrichment. However, it is worth noting that even though these methods suffer less from the dilution effects of pooling, they still rely on successful multiplication during enrichment to gain a target. A method that could be employed to achieve agar-based pooling is IMS. Since IMS can capture and concentrate the target

organism, and effectively wash away competitive microorganisms, it would greatly improve the detection of *Salmonella*. Also, IMS would be used for capture from a larger sample size than the 10 µl that is used to streak an agar plate. This would mean that the required CFU/ml would be much lower since theoretically, all the *Salmonella* present in the 1mlL would be captured and plated instead of a proportion of them. Incorporation of IMS would add complexity and cost to any such method but does offer a possibility of successfully pooling samples using only cultural methods. There are some examples in the literature that demonstrate the effectiveness of IMS pooling techniques (Wall *et al*., 2014), but they are not widely utilised in routine *Salmonella* detection in the UK (Personal observation).

As previously noted, most of currently described single stage protocols utilise a high temperature as part of the selective system used. Whilst the increased temperature does produce selective pressure (especially when combined with other components), other *Enterobacteriaceae* can proliferate at this temperature especially when initially present in high numbers. However, the temperature experiment demonstrated that there was no negative impact on *Salmonella* at 41.5 °C. Also, in most cases, greater suppression of competitive microorganism could be achieved at this temperature with the CNSX supplement. The CNSX supplement offers high selective pressure against common non-target organisms. However, the BPW formulation had a major impact on performance. The temperature experiment suggested that a less nutritionally rich BPW may be superior when dealing with high non-target organisms. The aBPW formulation seems to overcompensate for the selectivity of the CNSX supplement with high nutritional content, which promotes the overgrowth of the non-target organisms. *E. coli* appears to be more resistant to the sulfadiazine in the supplement at 41.5 °C possibly because of the expression of proteins or activation of survival systems (Arsene *et al*., 2000).

The temperature experiment showed that the aBPW + CNSX supplement was unsuitable as a single enrichment medium, due to overgrowth of non-target microorganisms. The LAB204 performed well but experienced a slight reduction in *Salmonella* recovery for some of the slower growing isolates,

such as *S.* Gallinarum. In the sensitivity experiment, LAB046 was used as a comparison as it had a higher nutritional content than LAB204, but less than aBPW. Matrix was also used as it presented a far greater challenge than pure culture. LAB046 completely out performed LAB204 in all tests and was much more likely to recover *Salmonella*. The external trail of this proposed system however did not achieve the same level of the performance as the ISO 6579 standard, thus it failed the validation test. The protocol seemed to be failing due to over growth of competitive microorganisms, most likely due to the nutritional content of the BPW. When paired with a less selective supplement and tested against a custom version of BPW (C3M3S3V1) and LAB046, LAB204 was successful in recovering *Salmonella* from all tests. The omission of cefsulodin from the enrichment supplement did not have a negative effect on performance, and the cefsulodin present in CASE was adequate to prevent false positive reactions. Both other versions of BPW failed to robustly detect the spiked *Salmonella* with multiple failures in most tests.

Since it became clear that LAB204 + NSM supplement offered the greatest chance of achieving a one broth enrichment strategy, further matrices tests were carried out. The first large scale matrices experiment (4.4.6) showed that LAB204 performed well as a BPW base with most matrix types. The custom C5Y5 (50 % casein peptone, 50 % yeast extract) BPW also performed well, but since LAB204 is a commercially available product it was easier to proceed with LAB204. The second large matrices experiment (4.4.6) showed that OBOP methodology performed similarly to the ISO methodology, except for the very low-level spike of the unpasteurised cheese. Both matrices experiments (4.4.6 & 4.4.7) had shown this matrix to cause difficulty for *Salmonella* detection. The experiments demonstrated the difficulty in OBOP methodologies. If the level of the target organism is low and the background microflora is high, it is difficult to achieve adequate multiplication of the target in a <18-hour time frame. The ISO method has an advantage over any single stage protocol because it has two enrichment steps. This means that any low level, potentially slow growing *Salmonella* have in excess of 37 hours to multiply to a detectable level. It is worth noting that in both matrices experiments, CASE was used as the secondary plating media alongside XLD, for the ISO 6579 methodology. When utilised

in this method it outperformed XLD in terms of sensitivity and selectivity, reinforcing the finding in Chapter 3. ISO 6579 contains data on the method validation studies carried out during the design of the standard method. The international interlaboratory study reports the sensitivity of the standard ISO 6579 method with various matrices materials, artificially spiked with high and low levels of *Salmonella* contamination. With complex matrices such as fresh cheese curd the standard ISO methodology only achieved a sensitivity of 74.3 % when inoculated with a low level (0.2 – 2.4 CFU per 25 g sample). However, with other matrices such as dried egg powder or poultry meat, almost 100 % sensitivity (98-100 %) was achieved at both the high and low level of artificial contamination. This demonstrates that even the extensive ISO 6579 methodology, with a dual enrichment step has its limitations. To create a single enrichment, single plating method to match the ISO standard is difficult because this format not only reduces the total opportunity of enrichment, but also reduces the number of tests carried out on a single sample to one

The main aim of this study was to determine an optimal BPW and selective supplement formulation for a single step enrichment protocol for *Salmonella enterica* from foodstuffs. This was achieved with the use of the commercially available BPW formulation from Lab M (LAB204) and a specifically paired selective supplement, containing novobiocin, sulphadiazine and mannitol (NSM). This combination was successful when used with a 10 µl subculture onto CASE, up to a detection limit of ~ 10 CFU for many food matrices types. The most challenging matrices for the single step enrichment detection method were either high fat, high nutritional content matrices (like soft cheese) or highly naturally colonised matrices (like chicken skin). The ability to pool samples, after enrichment, was evaluated but yielded unimpressive results. As such, it was deemed that pooling would be less sensitive than the standard cultural method described in ISO 6579. However, the single stage enrichment protocol, without pooling, appeared to be at least as sensitive to the standard method yet consumed less culture media and required 24 hours less to reach a result. This study also confirms that CASE can be used in a single stage enrichment protocol.

Chapter Five

Retail Produce Testing

5.1 Introduction

Salmonella is recognised as one of the most significant food-borne hazards in Europe. Due to successful surveillance and control programs, there has been a statistically significant decreasing trend for reported human clinical cases of salmonellosis for several years (EFSA and ECDC, 2017). However, over the past couple of years there has been a halt to this decreasing trend. This coupled with the rise of emerging monophasic serotypes (Petrovska *et al*., 2016) and multi-drug resistant strains (Rabsch *et al*., 2001), has led to increased concern over the control of this pathogen.

Pigs are one of the most common sources of human foodborne salmonellosis in Europe (De Knegt *et al*., 2015). *Salmonella* infections in pigs are mostly asymptomatic (Berends *et al*., 1996), however the presence is still problematic as there is a strong correlation between infected pigs and contaminated carcasses (Berends *et al*., 1997), which possibly will lead to human infection. This why a "farm to fork" risk assessment is widely observed when considering *Salmonella* in pigs (Hill *et al*., 2003). Of all the pork products mince tends to be one of the most common sources of *Salmonella* contamination (Stock and Stolle, 2001). This is likely because of the nature of production of the mince, which involves multiple processing and handling steps.

In the UK the criteria for the legally allowed limit of *Salmonella* in specific foodstuffs is legislated by European regulation 2073/2005. This regulation sets out the microbiological criteria for several common pathogens, as well as process hygiene criteria for various types of foodstuff. The requirement for *Salmonella* in many foodstuffs (including pork mince) is the absence of the organism in a 25 g sample, tested according to the standard method for *Salmonella* (ISO 6579). Furthermore, there are implemented surveillance testing requirements for the control of *Salmonella* in livestock during primary production. In the UK, legislation is dictated by European regulation 2160/2009, which states that pigs leaving for slaughter or carcases at the slaughterhouse must be tested for all *Salmonella* serotypes of public health significance. As per regulation 2073/2005 there is a requirement for the absence of *Salmonella* in the sample tested. In the case of foodstuffs, testing is done to protect the

public from this dangerous pathogen. If *Salmonella* is found, the foodstuff cannot be presented for sale. Primary production sample testing is carried out for surveillance data. If *Salmonella* is recovered, a collection of improvements and reviews will be carried out to reduce or prevent the incidence of the pathogen. The success of any microbiological surveillance program relies on the sensitivity and specificity of a detection methodology.

Chapter 4 described a one broth, one plate (OBOP) methodology as an alternative to the standard ISO 6579 workflow. The study demonstrated the ability to reduce the detection time to <48 hours with a single enrichment stage and a single chromogenic plate. This has advantages over the standard method, such as time to result and labour but it also has potential disadvantages, such as a reduction in sensitivity due to the shortened enrichment phase. The study detailed in Chapter 4 used real food matrices with artificial *Salmonella* spiking to challenge the method. As useful as this approach was to develop and challenge the new method for the isolation of *Salmonella* species, it cannot replace a study that uses naturally contaminated matrices. By using naturally contaminated matrices, a given method will be potentially challenged with a wide range of *Salmonella* serovars, as well as non-target organisms that may result in false positive results. Also, any *Salmonella* present will be subject to various levels of stress and sub lethal damage due to food processing techniques (Dodd *et al*., 2007).

The aim of this study was to test the alternative method developed in Chapter 4 in parallel with the standard ISO 6579 methodology, using naturally contaminated matrices. Studies on prevalence of *Salmonella* in retail meat have previously been carried out (Little *et al*., 2008a), however these studies commonly only used the standard ISO method for detection. This could have biased the ability to recover and detect certain atypical *Salmonella.*

5.2 Methods

5.2.1 Matrices source

Chapter 4 evaluated the OBOP methodology with various matrices that were deemed challenging, either due to microbial load or interfering characteristics of the matrix. For this comparative test of the proposed alternative method against the standard ISO 6579 method, different matrices were to be tested than those used in Chapter 4. The study in Chapter 4 used beef mince as representative of processed meat. In the EU, beef is mainly associated with *S.* Typhimurium zoonotic infections, whilst pork is associated with a wider range of serovars (EFSA and ECDC, 2017). Whilst the overall trend of reported *Salmonella* incidence from pigs in the UK has decreased over the past several years, there has been a substantial increase in monophasic stains of *S.* Typhimurium and multi-resistant isolates (Mueller-Doblies *et al*., 2013). These are both troubling new challenges that pose a new threat to public safety. Pork is also cited as a potential source of atypical *Salmonella* that would fail to result in positive identification on traditional media (Lin *et al*., 2014), as well as a potential reservoir of antimicrobial resistance (Rosengren *et al*., 2008). Another major source of salmonellosis in Europe is from consumption of contaminated poultry (EFSA and ECDC, 2017). The study detailed in Chapter 4 used chicken skin as a challenge due to the high bio burden found on this matrix. Due to historical and continued recent outbreaks linked to eggs (EFSA and ECDC, 2016), chicken and food products from chickens have been a major focus of food safety control measures. It is this focus which has led to a major decrease in prevalence of the pathogen in chicken flocks in the UK (O'Brien, 2013). Turkey food products are not implicated as often in zoonotic infections as chicken, but are still a significant reservoir for the pathogen (Antunes *et al*., 2016). In 2011-2012 there was a multi-country outbreak of *S.* Stanley in the EU, linked to contaminated turkey meat (EFSA and ECDC, 2012). Also interesting is the unusual distribution of *Salmonella enterica* serovars commonly isolated in turkey meat (Erol *et al*., 2013, Sanad *et al*., 2016), which differs greatly to other meat sources. A third major source of salmonellosis globally is the consumption of fresh produce. Many local authorities recommend the

consumption of at least 5 fruit and vegetables daily, some of which will be consumed raw. As such microbial contamination of such produce is highly dangerous. Since the devastating outbreak of *E. coli* O104:H4 in Europe (Soon *et al*., 2013), there has been greater concern regarding fresh produce as a source of pathogen infections. *Salmonella* is the most common bacterial pathogen linked to fresh produce (Callejón *et al*., 2015). There is also evidence to suggest that certain fresh produce, such as salad leaves are a much greater risk, because of the enhanced ability for *Salmonella* to colonise and grow on contaminated damaged leaves (Koukkidis *et al*., 2017). Unlike animal vectors, the contamination of fresh produce is mainly linked to the post-harvest processing prior to sale (EFSA, 2014b).

For this study pork mince and minced pork products (e.g. sausages) were chosen as the major focus of this comparative method study. For further variation, turkey mince was also included as well as a variety of fresh produce. Melon was highlighted as an important test matrices for fresh produce, due to repeated linked outbreaks (Walsh *et al*., 2014).

Various supermarkets in the North West of England were sampled as well as popular online supermarkets, to gain broader coverage of such meat being supplied. All the major top 10 UK supermarkets were sampled at some point in the study. For both pork and turkey mince it was common that supermarkets only had a single batch available, since it appears not be a high-volume stockholding in most supermarkets. So, it was common that 3 or more lots of the same batch were used for testing in this study. This was deemed acceptable since contamination would not necessarily be uniform throughout a minced product.

Care was taken when processing samples, to not cross contaminate via handling. Typically, the minced produce was presented in a 500g pack size, of which duplicate 25g samples were taken randomly from each pack. Sausages were also classed as a minced produce and the skin was removed from the meat prior to sampling. The melon samples tested were harvested from the skin of the melon and the flesh

was mostly excluded. All samples were recorded with at least a description, batch information, a source and country of origin.

After a first round of testing with pork mince and a second with fresh produce yielded no positive isolation of *Salmonella*, the scope was extended to include samples that had been produced to organic standards and/or were from animals that were reared outside. The ability of *Salmonella* to survive in the environment and difficulties in maintaining biosecurity measures means that outdoor reared pigs are at higher risk from transmission of infection (Jensen *et al*., 2006). It has been suggested that outdoor reared livestock are at greater risk to pathogens, such as *Salmonella* for several reasons. Outdoor reared animals may be at greater risk due to vectors such as rodents and birds (Meyer *et al*., 2005), which come into contact with the environment or the animals directly. These may carry and shed the pathogen, which can lead to transmission and infection of a herd. Organic reared pigs are also less likely to undergo disease prevention and intervention strategies such as vaccines and antibiotic treatment (Jackson and Cockcroft, 2007).

The sampling was conducted over a 15-month period starting in May 2016 ending in September 2017. The first round of testing that focused exclusively on retail pork mince samples was conducted between May and June 2016. The fresh produce testing was carried out in July 2016 and the final round of minced produced testing was carried out between March 2017 and September 2017. All samples were tested within their stated expiry dates, or best before dates for the fresh produce.

5.2.2 Testing protocol

All samples were tested in parallel with the ISO 6579 standard method (modified) against the alternative single stage enrichment described in Chapter 4. Each method tested a different portion of the same sample since the enrichment strategies were different. All isolated *Salmonella* were stored at -80 °C in cryopreservation vials (Protect, TSC) for further analysis.

5.2.2.1 ISO standard method

A 25 g of sample was homogenised via stomaching (60 seconds on high power) in 225 ml of buffered peptone water (BPW). This was then incubated at 37 °C for 18 hours, followed by a 1 ml subculture into 9 ml of Rappaport-Vassiliadis broth (RVS). This was then incubated at 41 °C for 18-24 hours followed by a 10 µl full plate streak onto xylose lysine deoxycholate (XLD) agar and chromogenic agar for *Salmonella* esterase (CASE) as described in Chapter 3. The agar plates were both incubated for 18-24 hours at 37 °C. Plates were then examined for typical colonies and any presumptive *Salmonella* were confirmed by latex agglutination (DR1108, Oxoid). The secondary enrichment in Muller-Kauffmann tetrathionate novobiocin (MKTTn) broth, as described by ISO 6579 was removed since this added extra complexity and rarely aided in the detection of *Salmonella* (personal observation). The total time for this methodology was three days. All culture media used (except CASE) were as formulated in ISO 6579.

5.2.2.2 Alternative method

The alternative method was the OBOP methodology detailed in Chapter 4. ISO formulated BPW was used with the NSM selective supplement (as described in 4.2.5). Briefly, 25 g of sample was homogenised via stomaching (60 seconds on high) in 225 ml of BPW + NSM supplement. This was then incubated at 41.5 °C for 18 hours followed by a 10 µl full plate streak onto CASE. CASE was incubated for 18-24 hours at 37 °C. The agar was then examined for typical colonies and any presumptive *Salmonella* were confirmed by latex agglutination (DR1108, Oxoid). The total time for this methodology was two days.

5.3 Results

The first part of the study tested 53 pork mince products, recording no *Salmonella* presence detected with either method. In total, the ISO method resulted in 19 presumptive positive samples, however on latex confirmation these were actually false negatives. The false negatives were exclusively due to

XLD and hydrogen sulphide producing non-*Salmonella*. The typical morphology of the false negative colonies was a rough colony with irregular colouring, part black and part yellow. This was clearly not the typical *Salmonella* appearance, but the ISO 6579 method requires all black colonies to be tested, by further biochemical or serological methods. The latex confirmation of the false positive colonies consumed a considerable amount of time. It is worth nothing that with the same ISO methodology enrichment, CASE did not yield any false negative colonies and was considerably easier to read. As neither method recorded any positive results it is impossible to compare performance, however the alternative method reached the same result a day earlier than the standard method. The testing of the fresh produce also yielded no positive results with either method. In total 30 samples of this type were tested and again XLD yielded 3 false positive results. Figure 5.1 demonstrates how by using CASE (in either methodology) a lower number of non-target colonies were recovered, compared to the same subculture on XLD. This made reading the plates easier and faster.

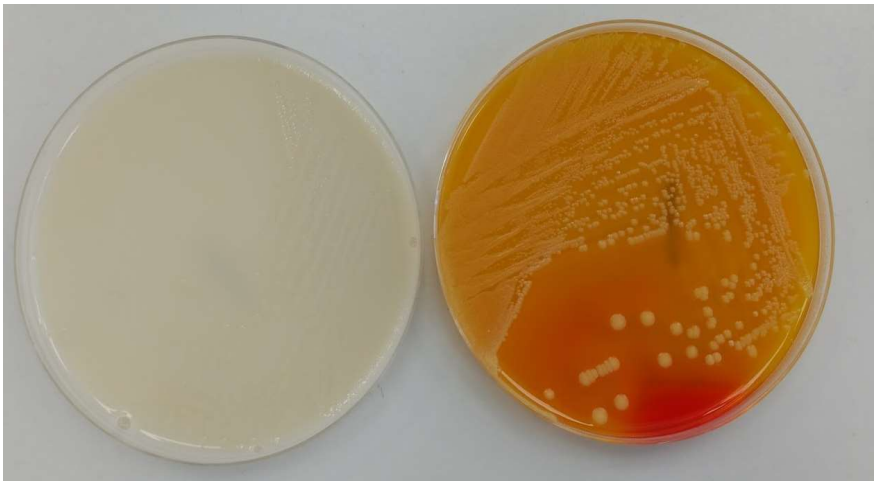


Figure 5.1 The resulting growth on CASE (left) and XLD (right) from the same RVS subculture. The XLD plate has heavy growth of a non-*Salmonella* lactose fermenting organism. The CASE plate has a much lower level of growth of the same organism but presents no colour.

The final part of the study shifted the attention to pork and turkey minced produce with a focus on outdoor reared and organic produce. The intention was to increase the chance of *Salmonella* contamination, as positive results were required to properly compare the methodologies. Not all

samples tested were outdoor reared and/or organic due to the availability of such material. In total 166 pork products were tested of which 4 resulted in recovery of *Salmonella*. Two of the positives were achieved with both methods; the other two were detected with the alternative method only. Two of the reported positives from pork mince were from the same batch of product. None of the positives were from confirmed organic or outdoor reared sources. In total, 44 turkey products were tested of which 6 resulted in recovery of *Salmonella*. Three of the positives were achieved with both methods, two were detected with the alternative method only and one was detected by the ISO standard method only. Two of the reported positives from turkey mince were from the same batch of product. One of the positives was from a confirmed outdoor reared source. Overall the alternative method had three positive deviations and one negative deviation. Since the number of positive deviations was greater than the negative deviations it can be assumed that the alternative method was at least as sensitive as the ISO methodology in this limited study. Table 5.1 summarises the full data set of results that can be found in Appendix 5.

Table 5.1 A summary of results from the comparison of the ISO and alternative method for confirmed isolation of *Salmonella* species from meat and fresh produce. The full table of results are available in appendix 5. Positive results were confirmed positives only (by latex agglutination).

Matrix	Samples	Positive Samples	Positive with ISO 6579 Method	Positive with Alternative Method	Positive with Both Methods
Pork Mince	125	3	2	3	2
Pork Sausage	41	1	0	1	0
Turkey Mince	38	6	4	5	3
Turkey Sausage	6	0	0	0	0
Fresh Produce	30	0	0	0	0
Total	**240**	**10**	**6**	**9**	**5**

In total 10 *Salmonella* isolates were recovered that were confirmed by latex agglutination testing, with the highest proportion from turkey mince (16%). All isolates were recovered between March 2017 and September 2017. These confirmed colonies were picked from the agar and enriched in tryptone

soy broth (LAB004, Lab M) and streaked for purity on tryptone soy agar (LAB011, Lab M). The cultures were then individually stored at -80 °C. After the study was complete the cultures were revived on tryptone soy agar and collected on charcoal Amies swabs (TS/5-10, TCS) for transport. The cultures are currently undergoing Illumina whole genome sequencing to determine the multi-locus sequence type (and associated serotype).

5.4 Discussion

The matrix testing described in this chapter primarily demonstrated the comparative performance of the alternative method against the standard ISO 6579 methodology, when using minced raw meat as a matrix. The alternative method was successful in recovering *Salmonella* where present in all but one example, where the ISO method alone achieved recovery. However, since the alternative method detected three isolates where the ISO method did not, it is likely that differences was due to sampling not performance. Since this study used two different primary enrichment methods, two different samples had to be taken from the same food matrix. Natural contamination of a food sample is likely to be non-homogenous, meaning some samples from the matrix could contain the pathogen whilst others may not. This issue could be prevented by homogenising a whole sample (500 g) and then taking the samples from the bulk. However, this is not common practice for routine *Salmonella* testing in the UK due to practicality. Also, homogeneity issues apply to the whole batch of mince produced. Thus, two separate 500 g packs of the same batch of bulk mince could have different contamination status. The two improvements of the alternative method over the standard, were time and specificity. The single stage enrichment of the alternative method, replaced the primary and secondary enrichment in the ISO method. As the final results of both methodologies were very similar, a day was saved for the alternative workflow to reach the same result. The standard ISO 6579 method requires dual plating of enriched samples. XLD is mandatory, but the user can select the second plate from preference. In the UK most laboratories select brilliant green agar (BGA) alongside XLD, due to cost and familiarity (personal observation). Both XLD and BGA rely on the inability of *Salmonella* to ferment

lactose, meaning atypical lactose fermenters may be missed. The chromogenic agar, CASE, was used as part of the standard method in this study. This improved the accuracy of the standard method as selectivity was superior and esterase detection, in the absence of β-glucosidase gave an excellent differential diagnostic to that employed in XLD. False positives are common with XLD as the hydrogen sulphide reaction is not completely specific to *Salmonella*. As observed in this study, food matrices often yield atypical colonies that present a black morphology. As the black hydrogen sulphide reaction is the key diagnostic for the target organism on XLD, all colonies that present such colouring must be classed as presumptive positive. CASE not only offers far greater selectivity than XLD but a more specific reaction, which leads to fewer false positives.

The later part of the study attempted to focus on outdoor reared and/or organic minced produce. This type of production was of interest as it is relatively underrepresented in the literature. The presence of *Salmonella* in pork is either due to infection during primary production or contamination during slaughter (Arguello *et al*., 2013). Whilst it is difficult to pinpoint the original cause of *Salmonella* contamination of foodstuffs, it is widely accepted that control measures during primary production are key to reducing prevalence of *Salmonella* in pigs and thus a reduction of salmonellosis in humans (Andres and Davies, 2015). Time to result is critical since time between transit from farm to slaughter is limited. Knowing the status of a herd would be beneficial to help prevent contamination at the slaughterhouse, as well as being able to release batch of product without known contamination. This is where a rapid cultural test would be advantageous. If the status of a herd is known prior to slaughter, the hygiene systems in a slaughter house can be more effectively managed to help reduce carcass cross contamination (Botteldoorn *et al*., 2003). Testing of processed meat products (e.g. mince) is also time critical because of the limited shelf life of the product. The longer a given detection method takes to report that status of a sample, the less time it is available for sale. Furthermore, if results yield presumptive positive *Salmonella* contamination which is in fact a false positive due to the diagnostic test, further time is wasted to confirm the status. Chromogenic identification from formulations like CASE would give a laboratory greater confidence in the identification.

A study investigating the prevalence of *Salmonella* in raw meat in Germany during 2008-2009, found 1.1 % - 1.8 % of pork meat products tested were positive for *Salmonella* depending on the methodology used (Meyer *et al*., 2010). Another study in Ireland found the prevalence of *Salmonella* in pork sausage to be generally less than 5 %, with the range dependant on source and time of year (Boughton *et al*., 2004). The combined prevalence of *Salmonella* of both matrices classed as pork products in this study, was 2.4 %. It is worth noting that two of the positive samples for pork mince came from the same batch of product. This level matches the expected incidence of *Salmonella* in pork products in the EU.

A study conducted with poultry samples in the UK during 2003-2005, found that 5.7 % of samples tested were positive for *Salmonella* (Little *et al*., 2008b). Recent summary reports from the European Centre for Disease Prevention and Control (ECDC), suggest the prevalence of *Salmonella* in turkey meat and its products to be 7.74 % (EFSA and ECDC, 2017). This study recovered Salmonella from 13.6 % of samples tested. The samples that were positive came from three different vendors/sources, showing that the detected contamination was not due to a single farm. However, two of the positives samples for turkey mince came from the same batch of product. Considering this and the relatively small number of samples tested (n=44), the level of incidence is close to that previously reported. Also, worth noting is the reported European prevalence is associated with more turkey meat products than just mince, thus it is not a direct comparison.

There is evidence to suggest that there is a recurrent trend of an increase in *Salmonella* prevalence in pigs during the summer months (Hald and Andersen, 2001). This is most like due to the rise in temperature in summer leading to an increase in the rate at which the bacteria can multiply. Furthermore, there is also evidence to suggest that higher temperatures lead to increased stress on the animal, resulting in an increased rate of shedding of *Salmonella* into the environment (Warriss, 1996). A similar trend of seasonal variation is observed in turkey with the summer months being linked to high prevalence (Erol *et al*., 2013). The study conducted in this chapter does not strictly match the

reported trend of higher prevalence of *Salmonella* during the summer months. The first sampling of pork mince was conducted during the summer of 2016 but yielded no positive samples. The final round of mince testing was conducted from spring to summer and half the positive samples were isolated during spring and the other during summer. To properly assess the reported increase during summer the winter months should be sampled, as well as increasing the sample size so significantly relevant statistics could be generated. This was beyond the scope of this study and further work would have to be carried out to investigate temporal patterns of incidence of contamination.

The testing of the fresh produce yielded no confirmed *Salmonella* isolates; however, the sample size was small (n=30). The fresh produce testing was included to challenge the alternative method with new matrix types. The alternative method cannot be properly evaluated against the standard method with these sample types, without any positives result. However, no false positives or other issues were encountered with fresh produce, when tested with the alternative method.

The serotyping of the isolates is still pending but it will be interesting to see if the serovars isolated, match those that are commonly prevalent in the corresponding matrices type. Further work should also include antimicrobial resistance profiling to attain the status of the isolates.

Due to the limited sample size and the issue of various retail packaging not being clear with the outdoor/organic status, it cannot be concluded that outdoor/organic minced produce has a higher level of *Salmonella* contamination. The availability of pork and turkey mince was generally lower compared to beef mince, and outdoor/organic produce further scarce. Thus, further work should be carried out to test a wider proportion of outdoor/organic minced produce, to investigate further.

Chapter Six

Immunomagnetic Capture and Concentration of *Salmonella*

6.1 Introduction

Immunomagnetic separation (IMS) is a method for specifically capturing and concentrating a target in a mixed solution, using antibody capture and magnetic particles. There are commercially available *Salmonella* IMS beads that have been used in cultural protocols, which have shown to be superior to the conventional ISO 6579 method (Cudjoe *et al*., 1994). Similarly, IMS beads have also been used to shorten the time to result and overall cost of testing, by using a combination of IMS and molecular detection methods (Koluman *et al*., 2012). Despite the published benefits of IMS for *Salmonella* detection the format is not widely used in the food testing industry, with most users opting for traditional (ISO) methods, or alternatively validated proprietary methods (personal observation). This can be attributed to several factors. Firstly, the cost of the IMS products alone is relatively high compared to traditional culture media. However, IMS can actually reduce cost by decreasing the amount of culture media required to reach a detection, in most cases by replacing the secondary selective enrichment step. It also can reduce cost by minimising labour and processing of a workflow. However, IMS is viewed as expensive since the IMS beads have a relatively high commercial cost per test. An IMS protocol would also use more plastic consumables and requires a magnetic separator (manual or automated), which would also add extra cost. Secondly, an IMS protocol adds more complexity and handling to what is normally a simple series of subculture steps. A manual method for IMS requires the dedicated attention of a user for around one hour, in which time multiple samples can be processed. Compared to the ISO 6579 workflow, after the initial sample prep, most other steps are proportionally much quicker. IMS methods may be unfamiliar to *Salmonella* testing laboratories, therefore time would have to allocated to train operatives. Finally, there are several reported problems with IMS including non-target carryover, ability to process only small sample sizes and loss of bound organisms due to washing and matric interference (Odumeru and Leon-Velarde, 2012).

The cost of commercial IMS products for *Salmonella* are on average £5 per test. The cost of commercially available IMS products can be attributed to the labour and knowledge to manufacture

them, as well as a high margin mark up on the material cost. Most of the costs associated with the consumable requirements is only initially high (i.e. the initial purchase of magnetic racks and washing systems), and these materials can be used for other foodborne pathogens, such as *E. coli* O157:H7 and other Shiga toxin-producing *Escherichia coli* (STEC). This reduces the cost burden on purely *Salmonella* testing. Regarding complexity, only minimal training is required to allow a competent microbiologist to perform the required techniques. Non-target carryover is a reported problem with some sources of *Salmonella* IMS products, but (from personal experience) this is heavily influenced by the bead type and binding chemistry used to attach the antibody. Most commercially available kits utilise a polystyrene coated bead, due to cost and simplicity. Whilst this is effective, the coating can lead to non-antibody related binding and non-favourable interaction with high fat matrices. There are other coatings available that are not as widely used, namely zirconium coatings. Coating with this transition metal results in a more inert surface and a heavier bead, which is not as highly influenced by high fat matrices. The issue of small sample sizes is connected to the average binding capacity for most IMS beads for *Salmonella*. It can be expected that a correctly performing IMS bead can bind and capture approximately 50 % of the target in solution. Therefore, if a given sample only contains very low numbers of *Salmonella* it is possible that an IMS bead may not capture enough to give a positive result with the detection method. Most IMS protocols for food pathogens utilise a 1 ml sample size and rely on sufficient multiplication during primary enrichment.

The aims of this study were to:

- Produce a low-cost *Salmonella* IMS bead that utilises a zirconium coated bead.
- Investigate the use of higher volume sample size IMS protocols.
- Demonstrate the functionality of the bead with matrices with high background levels of non-target bacteria.

6.2 IMS bead manufacture

6.2.1 Solid phase preparation

The raw magnetic particles (solid phase) used in this study were provided by Lab M Ltd. The beads had a ferric core with a zirconium coating and a particle size range of 1-8 microns. Large aliquots were stored at 2-8 °C in borosilicate bottles in water. Since the particles used had been static for many years in storage, the beads had to be properly resuspended in solution before coating.

First, a 2 litre bottle of unknown concentration of beads was rolled on a bottle roller shaker for 2 hours. A 100 ml aliquot of the suspension was removed and placed in a clean 500 ml polycarbonate beaker. The solution was made up to 500 ml with filtered reverse osmosis (RO) water and placed on a neodymium magnet, so that approximately 10 % of the beakers bottom was protruding over the edge of the magnet. The beads were then left for 5 minutes to collect against the bottom of the beaker that was in contact with the magnet. The supernatant was then aspirated using a vacuum pump drained into the sink. This was done on the side of the beaker that was protruding over the magnet, so that only the supernatant was aspirated not the magnetic particles. The beads were then resuspended in another 500 ml of filtered RO water. This washing step was repeated a further two times before resuspending in a final 500 ml of filtered (0.2 µm) RO water.

The washed suspension was then sonicated using a 700-watt ultrasonic processor using a ¾ inch probe. The probe was submerged in the solution with care so that it did not touch the bottom of the beaker. The ultrasonic processor was run on 80 % power for 90 seconds. Then the beads were washed as previously described. This was repeated two further times before resuspending in a final 500 ml of filtered RO water, in a 500 ml sterile Duran-type bottle.

6.2.2 Dry weight analysis

To calculate the concentration of beads in solution, dry weight analysis must be carried out. This was done by removing all the water from a sub sample and weighing the beads. Dry weight analysis is a

destructive test so only a small sub sample is used. The concentration is important for all production steps and for performance of the final product, so dry weight analysis is carried out at multiple points during production.

The sonicated suspension described in 6.2.1 was rolled for 30 minutes on a bottle roller shaker. The weights of five separate 5 ml Bijou bottles were recorded up to four decimal places using an analytical balance. Five 1 ml aliquots were removed with a pipette and placed into five separate 5 ml Bijou bottles. The samples were then washed with RO water two times in the same manner as described in 6.2.1, by concentrating the beads onto a magnet and removing the supernatant. After the second wash, the beads were not resuspended but placed into a drying oven set at 100 °C for at least two hours. The samples were then removed from the oven and allowed to cool to room temperature. All the samples were then weighed up to four decimal places using an analytical balance. The weight of the empty Bijou bottles was subtracted from the weight of the Bijoux plus the dried beads to calculate the g/ml in the original bead suspension.

6.2.3 Buffer / solutions production

Various buffers and solutions were required to produce the IMS beads.

6.2.3.1 - 10 mM pH 7 phosphate buffer

Di-sodium hydrogen orthophosphate 2-hydrate ($Na_2HPO_4.2H_2O$) and potassium dihydrogen phosphate ($KH2PO_4$) were added to RO water to create the phosphate buffer. For 1 litre of buffer, 0.635 g of KH_2PO_4 and 0.950 g of $Na_2HPO_4.2H_2O$ were dissolved in RO water. When both components had fully dissolved the pH was checked using a calibrated pH meter. If the buffer was pH 7.0 ± 0.1 the solution was sterilised by filtering through a 0.2 µm membrane into a sterile vessel.

6.2.3.2 - 1 % Polyethylenimine (PEI) solution

To produce 1 litre of 1 % PEI solution, 20 g of a 50 % aqueous solution of PEI was added to a sterile Duran-type bottle. Filtered RO water was then added so that the final displayed mass was 1000 g. This

solution was then roller mixed on a bottle roller until the PEI had completely dissolved. This solution was not sterilised but just used on the day of preparation.

6.2.3.3 - 10 % Glutaraldehyde solution

To produce 1 litre of a 10 % glutaraldehyde solution, 0.4 l of 25 % glutaraldehyde solution (grade II, Sigma Aldrich G6257) was added to 0.6 l of filtered RO water in a clean Duran-type bottle. This was mixed on a roller shaker for at least 10 minutes. This solution was not sterilised but just used on the day of preparation.

6.2.3.4 – Bovine Serum Albumin (BSA) solution 10 mg/ml

To produce 1 litre of 10 mg/ml BSA solution, 10 g of BSA (≥96 % lyophilised powder, Sigma Aldrich A2153) was added to 1 litre of 10 mM pH 7 phosphate buffer (as described in 6.2.3.1) in a clean Duran-type bottle. This was dissolved by rolling and sterilised by filtering through a 0.2 µm membrane into a sterile vessel.

6.2.3.5 – IMS diluent

The final IMS beads, once activated and coated are stored in a blocking and preservative solution. This is to prevent microbial contamination and maintain them on storage. The buffer consists of a dual phosphate buffer (using the same components as 6.2.3.1), sodium chloride (NaCl) and potassium chloride (KCl) to maintain the osmotic balance, sodium azide (NaN_3) as a preservative and BSA as a blocking agent.

Table 6.1 List of components and concentrations required to produce 1 litre of IMS diluent. All components were added to 1 litre of RO water.

Component	g/l
Na_2HPO_4	1.19
KH_2PO_4	0.22
NaCl	8.0
KCl	0.2
NaN_3	0.9
BSA	10

All components specified in Table 6.1 were dissolved in 1 litre of RO water. If the buffer was pH 7.4 ± 0.1 the solution was sterilised by filtering through a 0.2 µm membrane into a sterile vessel.

6.2.4 Solid phase activation

To be able to coat the magnetic particles in a specific antibody, they must first be activated to bind the antibody. This was done by modifying the bead surface so that antibody is captured and bound to the bead. There are various chemistries and protocols that can do this, but for this application an amino modified covalent coupling (Reichlin, 1980) was employed. One of the most popular strategies used for this (and similar) application(s) is to streptavidin coat the beads, to cross react with biotin labelled antibodies. Whilst this process is much simpler and more user friendly (less toxic), it yields a IMS beads which is much more prone to nonspecific binding than the covalent coupling chosen for this study (personal observation, data not shown).

The prepared beads described in 6.2.1 were resuspended by rolling for 30 minutes. Using the concentration established in 6.2.2, an aliquot was removed using a clean, calibrated, graduated cylinder. The aliquot was measured so that it contained 1 g of beads. The aliquot was then washed twice as described in 6.2.1, by concentrating the beads onto a magnet and removing the supernatant. The beads were then resuspended in 20 ml of filtered RO water in a sterile 300 ml polycarbonate container, to which 20 ml of PEI solution (as described in 6.2.3.2) was added. This solution was roller mixed for 1 hour, then washed three times as previously described. The beads were then resuspended in 20 ml of filtered RO water in a new sterile 300 ml polycarbonate container, to which 20 ml of glutaraldehyde solution (as described in 6.2.3.3) was added. This solution was roller mixed for 1 hour, then washed three times as previously described. The beads were then resuspended in 300 ml of filtered RO water in a new sterile container.

The concentration was then attained by dry weight analysis (as described in 6.2.2).

6.2.5 Pre-prepared antibody preparation

An affinity purified polyclonal antibody raised to *Salmonella* common structural antigens was purchased (BacTrace® Anti-Salmonella CSA-1, Seracare 01-91-99). The exact antigenic target was proprietary to the manufacturer, but the literature described that it was isolated from a serum pool of goats immunised with different serotypes of *Salmonella*. As such it claims specificity to *Salmonella* serotypes of groups A, B, C ,D & E (according to the Kauffman-White classification (Grimont and Weill, 2007)).

The antibody was supplied in a lyophilised format containing 1mg of antibody. This was reconstituted in 20 ml of phosphate buffer (as described in 6.2.3.1) and filter sterilised (0.2 µm membrane) into a sterile universal tube. This was done no more than 10 minutes before coating of the activated beads to reduce the chance of precipitation.

6.2.6 Solid phase coating and blocking

Coating and blocking of the activated magnetic particles consists of three steps. Firstly, mixing with the antibody to bind it. This is followed by blocking the remaining active sites with BSA to prevent nonspecific binding. Finally, the reaction is stopped by adding borane *tert*-butylamine (BTB) complex which effectively reduces the aldehyde functional group. Using glutaraldehyde to covalently immobilise proteins came about from research regarding leather tanning, which highlighted the ability of aldehyde to cross link with the skin proteins in leather (Bowes and Cater, 1965). The aldehyde group reacts predominantly with the Ɛ-amino groups of proteins (Habeeb and Hiramoto, 1968), in this case the primary amines on lysine residues present on the Fc region of IgG antibodies. It is possible this will result in a non-functional orientation of the antibody depending on the makeup of a given antibody (Welch *et al*., 2017). However, for many antibodies (including the source used here) this kind of immobilisation results in a strong, correctly orientated, immobilised antibody that has low non-specific binding.

The 1 g of activated beads (described in 6.2.4), was resuspended by rolling for 30 minutes. It was then washed twice in phosphate buffer (6.2.3.1) in the same manner described in 6.2.1. The beads were then resuspended in 20 ml of phosphate buffer. The antibody solution prepared in 6.2.5 was then added to the vessel and roller mixed at room temperature for one hour. Then 20 ml of the BSA solution (6.2.3.4) was added to the vessel and further roller mixed at room temperature for one hour. A 10 mg/ml solution of BTB (Sigma Aldrich 180211) was made in 75 % N,N-Dimethylformamide (DMF) and dissolved by brief vortex mixing. 0.4 ml of the BTB/DMF solution was added to the coating vessel and further roller mixed at room temperature for one hour. The beads were then washed 3 times in 40 ml the IMS diluent (as described in 6.2.3.5) as per 6.2.3.1. The beads were then resuspended in 40 ml of the IMS diluent in a sterile 300 ml container.

6.2.7 Dilution to working strength

The beads have a target concentration of 5 mg/ml when being used for IMS. The coating and blocking stage is done at a higher concentration so they must be diluted to reach the correct working strength. This is simply done by adding more IMS diluent to the vessel so that the solution contains 5 mg/ml of beads. A dry weigh analysis was carried out (as per 6.2.2) to attain the current concentration. A calculation was then carried out to attain how much extra IMS diluent is required.

6.3 IMS bead performance testing

To test the functionality of the produced IMS beads, they must be challenged with their ability to specifically bind *Salmonella.* This is done by mixing the beads with a known concentration of *Salmonella*, washing to remove unbound organisms, plating onto an agar and counting the recovered colonies. A non-target organism, *E. coli* was also tested in the same manner to test the specificity of the IMS beads. 20 µl of IMS beads were used per 1 ml of test sample.

S. Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076 and *E. coli* ATCC 25922 were grown overnight in buffered peptone water (BPW) at 37 °C. The cultures were then diluted in maximum recovery diluent (MRD), so that 0.1 ml of the suspension contained approximately 10-100 CFU. 1 ml of this dilution was then added to a 1.5 ml microcentrifuge tube with 20 µl of the IMS beads (produced in 6.2). 0.1 ml was also spread onto a tryptone soy agar (TSA) plate and incubated for 18 hours at 37 °C. The sample/bead solution was mixed on a rotatory tube mixer (Stuart, SB3) at 25 RPM for 30 minutes. During this mixing step, the wash buffer was produced. The IMS wash buffer was simply phosphate buffered saline (PBS) tablets x 5 (Sigma Aldrich 79382) and 0.5 g/l Tween® 20 (Sigma Aldrich P1379) in 1 litre of RO water. After all components were dissolved by roller mixing, the buffer was sterilised by membrane filtration (0.2 µm). After mixing of the bead/sample, the tubes were then placed in a magnetic rack (Dynabeads™ MPC™-S). The beads were left to collect on the magnet for 3 minutes (as shown in Figure 6.1). The supernatant was then removed by pipette and replaced with 1 ml of sterile wash buffer. This washing process was repeated two more times, then the beads were suspended in

0.1 µl of sterile wash buffer. The solution was then spread onto TSA and incubated for 18 hours at 37 °C. All plates were counted the following day and percentage recovery was expressed. To calculate the percentage recovery, the counts of the pre-IMS plates were multiplied by 10 as 100 µl had been originally plated, compared to the 1 ml that had been used in the IMS procedure. To have confidence in the result, the performance testing was performed with four replicates.

Figure 6.1 IMS beads collected on the wall of a microcentrifuge tube due to the magnetic field of a magnetic separator rack.

6.4 IMS protocol challenge

6.4.1 Background

After the successful manufacture and performance testing of anti-*Salmonella* IMS beads, matrix testing was carried out to evaluate performance of the beads. Initial work demonstrated that porcine faeces was one of the most difficult matrices to test for many reasons. Firstly, samples were not consistent in terms of physical characteristics such as pH, moisture content and variability of undigested material. This led to variable environments in liquid culture between experiments and thus variable success in standard culture methods. Also, porcine faeces had a consistently high bio-burden which was predominantly Gram negative, lactose fermenting / β-galactosidase positive and often led to out competing of *Salmonella* using traditional methods. Finally, when tested by IMS, the matrices

would ‘stick’ to the beads/reaction vessel. Even with increased washing steps, this would lead to high non-specific binding and subsequent crowding and/or masking of the bound target organism on solid media.

To reduce the matrix effect, filtered stomacher bags were evaluated. These bags are standard stomacher bags but with the addition of a semi-permeable membrane or strainer, that allow microorganisms to pass through the membrane to a compartment that can be sampled via a pipette. Matrix debris is retained on the other side of the membrane greatly reducing material carryover. The two types of bags tested in this experiment were the Interscience BagPage® + and Interscience BagFilter® P. The BagPage has a micro perforated filter down the middle of the bag which effectively creates two equal compartments. The BagFilter has a woven strainer down one side of the bag creating a large sample compartment and a small sampling compartment. Both function in the same way, in that the media and sample are added to the bags and stomached for the required amount of time. It is during this process that the flora is homogenised throughout both compartments. Liquid and thus microorganisms can move freely across the membranes allowing for a representative sample to be taken.

The major difference compared to traditional methods, besides the IMS aspect of the proposed protocol is the time samples are enriched. Due to the ability of IMS to capture and concentrate a target organism, a prolonged enrichment to generate cell numbers is not needed. This is because traditionally users rely on high cell density after enrichment to guarantee that the target of interest is present in the subculture volume, which is greatly smaller than the total enrichment volume e.g. 1 ml subculture from 225 ml enrichment. With IMS the target can be captured and concentrated from a larger volume without the need to carry over non-target organisms and matrices to the second stage. It is proposed therefore that samples should be enriched for 4 hours to allow for resuscitation of stressed cells, but also allow same day processing of samples.

The other aspect to this protocol is concerned with sample size. Traditionally IMS is carried out in 1 ml volumes for convenience of manual washing. However, this is a problem if a traditional overnight enrichment is not carried out, since an original low level of target may not be present in a high enough level to be detected in a 1 ml sample. If a 25 g sample contains 100 viable *Salmonella* and it is homogenised in 225mL, 1mL will likely contain less than 1 cell. Initial work on this protocol showed that a low-level spike of Salmonella with a 3-hour enrichment followed by IMS from 1 ml, failed to yield positive *Salmonella* on a selective agar. The sample size was subsequently increased to 10 ml in adapted glassware for magnetic immobilisation to improve the chance of low level capture and detection. By using 10 ml it is still possible to concentrate down to a 100 µl final volume for agar plating. This is done by sequential washing and reducing steps in new reaction vessels. Coincidentally by reducing the volume sequentially and transferring to smaller 1.5mL plastics, lower carryover of non-target unbound was observed in earlier work.

6.4.2 Materials

Fresh porcine faeces was obtained from a local pig farm and tested on the day of collection.

Buffered Peptone Water (BPW), Rappaport-Vassiliadis Medium (RVS), Maximal Recovery Diluent (MRD), Tryptone Soya Agar (TSA) and *Salmonella* ABC Agar were all provided by Lab M Ltd and prepared to manufacturer instructions. Anti-*Salmonella* IMS beads and PBS + 0.05 % Tween® 20 wash buffer was used as described in 6.3. Microcentrifuge tubes with screw cap (1.5 ml) and 10 ml Leighton tubes were used for the IMS capture and washing steps, with different magnetic separators suitable for both vessels. A rotary tube mixer (Stuart, SB3) was used to mix IMS reaction tubes. A Seward stomacher® was used to homogenise samples in both sample bags (Interscience BagPage® + & Interscience BagFilter® P)

S. Typhimurium ATCC 14028 was grown in BPW and incubated at 37 °C for 18 hours then serially diluted in MRD so that 100 µl of solution contained approximately 100 cells. Control plating of the same dilution was carried out on TSA incubated at 37°C for 18 hours to enumerate.

6.4.3 Method

6.4.3.1 Natural contamination testing

Since the porcine faeces used possibly could contain *Salmonella* which could mask any failing of the method to detect the spiked organism, all matrix material was screened for *Salmonella* by traditional methods. This involved an 18-hour enrichment at 37 °C of a 25-g sample in 225 ml of BPW followed by a 0.1 ml subculture into 9 ml RVS incubated at 41.5 °C for 24 hours, followed by a 20 µl streak onto ABC agar. The matrix material was tested in triplicate. ABC agar detects *Salmonella* species by the presence of α-galactosidase in the absence of β-galactosidase. *Salmonella* appear green on the agar, whilst all other organisms are either black or colourless.

6.4.3.2 Sample size

The porcine faeces collected was segregated into 12 separate 25 g samples. Six samples were tested using the BagPage® +, and 6 were tested using the Interscience BagFilter® P. All tests were identical except for the two different filter stomacher bags.

6.4.3.3 *Salmonella* spike

Artificial spiking of the matrix material was carried out prior to stomaching, by depositing 50 µl of the culture described in 6.4.2 onto the surface of the matrix.

6.4.3.4 Rapid IMS protocol

1. Add 225 ml of pre-warmed (37 °C) BPW into a stomacher bag
2. Add 25 g of sample matrix
3. Stomach mixture for 60 seconds on the high setting
4. Incubate mixture at 37 °C for 4 hours
5. Take 10 ml sample from filter compartment and add to Leighton tube
6. Add 100 µl of Anti-Salmonella IMS Beads
7. Mix (orbital) sample for 30 minutes
8. Immobilise beads on magnet (at least 5 minutes) and dispose of remaining liquid
9. Add 10 ml of wash buffer and repeat step 8

10. Suspend beads in 1.5mL of IMS Wash and collect in a 1.5 ml Eppendorf tube
11. Immobilise beads on magnet (at least 3 minutes) and aspirate remaining liquid
12. Add 1 ml of IMS Wash and repeat step 11
13. Add 1 ml of IMS Wash and repeat step 11
14. Suspend beads in 100 µl of IMS wash
15. Surface spread solution onto a dry ABC agar plate
16. Incubate plate for 18-20 hours and inspect for typical colonies

6.4.3.5 ISO 6579 and control testing

Supplementary to the rapid IMS protocol, the standard ISO 6579 method was carried out (as described in 6.4.3.1) with the shortened (4 hours) and standard (18 hours) primary enrichment step from the same samples. This was to compare the potential benefit of the rapid IMS protocol regardless of the time saving it offered. Alongside the 4-hour IMS sampling step, 100 µl of the same enrichment was streaked directly onto a ABC plate. This was to attain if *Salmonella* could be detected without the IMS step at this stage.

6.5 Results

6.5.1 IMS bead performance

The IMS beads were tested for their ability to capture *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076 in pure culture, by comparing the number of organisms present before enrichment to numbers present after the IMS capture protocol. For the beads to be viable as a test it was expected that they achieve around 50 % capture of the available target organisms. The results and calculated percentage recovery is displayed in Table 6.2. The beads captured almost 50 % of the available target organisms present, whilst achieving very low (<2%) non-specific binding with an *E. coli* type strain.

Table 6.2 Recovery counts and calculated percentage recovery of IMS *Salmonella* beads produced as described in 6.2.

S. Typhimurium ATCC 14028	Number of colonies pre-IMS (CFU/ml)	Number of colonies post-IMS (CFU/ml)	
1	921	401	
2	944	488	
3	1021	457	
4	978	461	Percentage Recovery
Mean	966	452	46.8%
S. Enteritidis ATCC 13076	Number of colonies pre-IMS (CFU/ml)	Number of colonies post-IMS (CFU/ml)	
1	1084	489	
2	1079	523	
3	1088	477	
4	1013	503	Percentage Recovery
Mean	1066	498	46.7%
E. coli ATCC 25922	Number of colonies pre-IMS (CFU/ml)	Number of colonies post-IMS (CFU/ml)	
1	1114	22	
2	987	17	
3	1099	8	
4	1130	25	Percentage Recovery
Mean	1083	18	1.6%

6.5.2 Matrices testing

The IMS beads were challenged with the recovery of a low-level *Salmonella* spike in the presence of high level non-target organisms from naturally contaminated porcine faeces. Standard ISO 6579 testing of the porcine faeces detected no natural *Salmonella* contamination. Enumeration of the *Salmonella* spike showed that the inoculation level was 84 CFU. The results of the matrices IMS

challenge test as described in 6.4.3 are displayed in Table 6.3. All samples tested successfully recovered *Salmonella*, with all ABC agar plates yielding positive α-galactosidase positive, green *Salmonella* colonies. Black β-galactosidase positives colonies were also recovered but they did not prevent the positive identification of *Salmonella.*

Table 6.3 Plate counts on ABC agar from two stomacher bag types from the testing protocol described in 6.4.3.

BagPage	Black (non-target) Colonies	Green (*Salmonella*) colonies
1	4	12
2	6	11
3	2	7
4	2	13
5	11	13
6	2	5
BagFilter	**Black (non-target) Colonies**	**Green (*Salmonella*) colonies**
1	15	8
2	13	13
3	12	8
4	15	7
5	11	12
6	17	11

All direct streaks of the 4-hour enrichment failed to yield any observable green colonies. Any *Salmonella* present were obscured by the present of a high level of black colonies. All samples were also put through secondary RVS enrichment after both the shortened (4 hours) and standard (18 hours) primary enrichment. All tests successfully recovered *Salmonella* but had high levels of non-target organisms also present on the ABC agar. The BagFilter stomacher bag generally had a higher level of black colonies on subculture after RVS enrichment, compared to the BagPage stomacher bag. Visually, the subculture samples taken from the BagPage stomacher bag appeared to have fewer visible matrix particulates, compared to the BagFilter stomacher bag.

6.6 Discussion

The production of a low cost IMS bead product for *Salmonella* was successful, as the approximate material cost of the bead per test was £0.20 per test. The initial performance testing demonstrated the beads functionality, successfully recovering *Salmonella* type strains. The recovery of the non-target *E. coli* was very low (<2%). If the samples had been plated on a superior *Salmonella* selective agar such as CASE (as described in Chapter 3), it is likely that they would have yielded no non- target colonies.

The rapid IMS protocol, using a short pre-enrichment step followed by IMS and agar plating, was successful in recovering a low-level spike of target organism with a low level of non-target recovery. The time to result for the IMS protocol was approximately 22 hours, whereas sub culturing into RVS then plating onto ABC yielded a result in approximately 45 hours. Direct subculture onto ABC from initial pre-enrichment did not yield a positive result thus no time to result is recorded. The BagPage stomacher bag seemed more efficient at reducing matrix carry over to the test sample than BagFilter. This resulted in lower non-target organisms in the RVS subculture streak, however the IMS protocol due to its specificity and washing steps did not suffer from any non-target issues from either bag. The slight improvement of the BagPage stomacher bags is most likely due to their filter membrane. It appears that this filter membrane more effectively retains a matrix like faeces, compared to the membrane in the BagFilter stomacher bags. Carry over of any matrix material is detrimental to the performance of an IMS test. This is because the IMS beads can get stuck in matrix material and stick them to the vessel wall during magnetic immobilisation, reducing the efficiency of the washing step. The direct subculture streak from the bags after 4 hours pre-enrichment failed to detect the *Salmonella* spike. This is mostly likely because the level of non-target organisms present in the enrichment at four hours was far greater than the level of *Salmonella*. The 4-hour enrichment is not sufficient on its own to detect *Salmonella* on an agar diagnostic test. The ISO 6579 methodology

successfully recovered the *Salmonella* spike, even with a shortened pre-enrichment step. However, this took 44 hours which is significantly longer than 22 hours achieved by the rapid IMS protocol.

The success of the rapid IMS protocol (as described in 6.4.3.4) demonstrates the potential of IMS to be used as tool to reduce the time to result, due to its ability to concentrate and specifically capture a microorganism. However, due to popularity and familiarity of the ISO 6579 methodology it is likely that a proposed IMS step would meet resistance to uptake, in the commercial testing market. To be successful in gaining widespread implementation, IMS methods seem to have to find a niche where without them the existing method is poor. For example, IMS testing protocols are heavily present in *E.coli* O157:H7 methodologies, as without the IMS step the ability to detect low level contamination is much lower (Wright *et al*., 1994). As the ISO 6579 method is as sensitive as the proposed rapid IMS method in this study, there would be no reason to adopt the IMS method despite the time saving.

The IMS protocol would need to be extensively tested with a large array of matrices types and with a lower artificial spike, to properly judge its performance against the standard method. Also, due to the extensive diversity of *Salmonella* spp., more organisms should be tested for their antigenic capture efficiency. Personal communication with other researchers that have also used the same polyclonal antibody as described in 6.2.5., suggested that an immunological test may suffer from poor recovery of Group E *Salmonella*. There is also literature evidence of the variability of immunological methods for *Salmonella*, specifically Group E, such as *S.* London (Cudjoe *et al*., 1995). Variability in the reactivity of a *Salmonella* IMS product is an issue, however it is worth noting that the ISO 6579 cultural method also has significant limitations. In conclusion, I believe the most effective application of IMS for *Salmonella* would be for a specific group or serovar/s that traditional methods can often fail to isolate, such as *S.* Typhi (Hammack *et al*., 2008).

Chapter Seven

Salmonella Typhi Isolation Methods

7.1 Introduction

Salmonella enterica subsp. *enterica* Serovar Typhi (*S.* Typhi) is the leading cause of enteric fever responsible for millions of cases and over 100,000 deaths each year (Crump and Mintz, 2010). *S.* Typhi is a highly adapted human specific pathogen with an extensive mechanisms for persistence and survival in its host (Parry *et al*., 2002). *S.* Typhi differs from many other *Salmonella* serovars which are associated with food poisoning and gastroenteritis, as it infects via person-to-person rather than a zoonotic source. The infection is spread by ingestion of contaminated food and water by the faecal-oral route. Modern effective sanitation and hygiene systems in Europe mean that disease due to *S.* Typhi is exceptionally rare, with most cases due to returning travellers from high risk areas (Dave and Sefton, 2015). However, in low and middle income countries the disease burden is high due to inadequate access to safe drinking water and effective sanitation (Steele *et al*., 2016).

Prevention strategies for typhoid fever include improved water systems and sanitation, education on hygiene and vaccination. There are three vaccines available for typhoid fever, however they have drawbacks such as adverse reactions, the requirement of repeated dosing and stability issues (Paterson and Maskell, 2010). One of the vaccines is Ty21a, a live attenuated *galE* mutant of *S.* Typhi Ty2 (Cryz *et al*., 1988). This organism is classed as a containment level 2 organism compared to *S.* Typhi generally, which is containment level 3. Most microbiological laboratories in the UK are either containment level 1 or 2, meaning they can only handle organisms that can cause disease but are unlikely to spread to the community and effective treatment is available. Since *S.* Typhi can cause serious disease and may spread to the community, work on the organism in the UK is restricted to specialist containment level 3 laboratories.

An area where a large concentration of research is carried out on invasive *Salmonella* is Malawi, in sub-Saharan Africa. Studies carried out at the Queen Elizabeth Central Hospital in Blantyre (Malawi) have observed a relatively high level prevalence of typhoid fever (Feasey *et al*., 2010). Recurrent epidemics of *S.* Typhi have also been associated with emerging drug resistance (Pitzer *et al*., 2015).

Due to the rising threat of antimicrobial resistance to the commonly employed drugs (Kariuki *et al*., 2015), it is ever more important to properly deploy resources to improve preventative measures such as vaccines and sanitation programs.

Dr Nicolas Feasey, a researcher and physician at the Liverpool School of Tropical Medicine (LSTM) is the coordinator of a project to improve *S.* Typhi detection methods in Malawi. The research is funded as an initiative of the Bill & Melinda Gates foundation. The project aims to improve detection methods of *S.* Typhi so that live cultures can be isolated and confirmed from environmental sources. This would ensure that intervention strategies are focused on the areas where the organism is of greatest significance and thus, help reduce the burden of disease by reducing the incidence of infection via targeted sanitation programs. Currently there are difficulties in isolating *S.* Typhi from the environment and molecular based methods appear to be inefficient and, in some cases, inaccurate. Dr Feasey stated that molecular methods currently used in the field were reporting strong positives for some water samples, where no organism was being recovered and no human cases were reported. Therefore, culture-based methods are preferred. Furthermore, isolation of viable *S.* Typhi is a clear indication of the presence in the environment and a public health risk. The plan was to spend time optimising the methodology in the UK at LSTM and Public Health England (PHE), before implementing the methodology in the field in Blantyre. The trial, if successful will be further implemented across Africa and other areas affected by typhoid fever.

In collaboration with Dr Feasey's group, current methodologies for the isolation and identification of *S.* Typhi were evaluated. CASE (as described in Chapter 3.) was sent to PHE for Rory Miles (under the supervisor of Dr Nicola Elviss) to trial with *S.* Typhi isolates. In the meantime, the vaccine strain, Ty21a was acquired from LSTM to carry out work on immunological methods. The concept proposed by Dr Feasey was to use immunomagnetic separation (IMS) beads to capture and concentrate *S.* Typhi from water sources before isolating and identifying on culture-based diagnostics. Initial feedback from PHE was that CASE did not support the growth of wild type *S.* Typhi isolates as well as traditional

formulations such as bismuth sulphite agar. *S.* Typhi had never been tested on CASE during the development because of biosafety considerations, however it was known to possess esterase thus the issue must have been due to the growth promotion or selectivity of the agar. Access to the containment level 3 laboratories at PHE (Colindale) was organised so that time could be spent working with wild type isolates of *S.* Typhi. Time was limited thus, work would be carried out at the laboratories of Lab M / University of Liverpool first in order to maximise the use of the PHE labs.

The aims for this study were:

- To produce a specific *S.* Typhi IMS bead.
- To test the produced IMS beads and the previously created generic anti-*Salmonella* IMS bead (Chapter 6) for their ability to capture Ty21a.
- Investigate the reported failure of CASE to recover *S.* Typhi.
- If necessary, modify CASE to improve the growth and identification of *S.* Typhi.
- Test IMS beads and Chromogenic agar against a wide range of *S*. Typhi phage types, in order to develop a new methodology for *S.* Typhi isolation.

For clarity, all work described in this chapter was carried out by the author.

7.2 Methods

7.2.1 Purification of antibody for *S.* Typhi specific IMS beads

7.2.1.1 Antibody source

Referring to the antigenic formula of *S.* Typhi (9,12,[Vi]:d:-:[z66]) (Balows *et al*., 2012), antisera was purchased from SSI Diagnostica (Statens Serum Institut, Denmark). The candidate antisera chosen were O9, O12 and Vi, as previous experience had shown that somatic (O) antigens were often the most successful targets for IMS capture. Also, these specific antisera were much more available at a relatively low cost. The O and Vi antisera are used for serological confirmation of *Salmonella* by slide agglutination. So, the antisera are very likely to have good activity against whole live cells, as required

for an effective IMS antibody. However, to be utilized as an antibody source for an IMS bead the antisera required purification.

7.2.1.2 Protein A column

Staphylococcal protein A is one of the most long-standing methods of antibody affinity purification. Protein A can bind to the heavy chain constant region (Fc) of immunoglobulin G (IgG) (Hober *et al*., 2007). Antisera can be purified into concentrated IgG fractions by affinity chromatography via a protein A column. The methodology involves loading an antibody solution onto a column at a neutral pH. The IgG antibodies bind to the protein A and all other material is washed away. The antibody is then eluted from the column by using a low pH elution buffer. The protein fraction is then detected by UV absorbance (280 nm) and collected.

Prosep A, high capacity (Merck, Germany) was used to build a 5 ml Protein A column. Prosep A is controlled pore glass with a recombinant native protein A ligand, which was used as the chromatography media. A 10mm fluorinated ethylene propylene (FEP) glass column was used, with 5-micron polyethylene frits and various O ring and end caps and connectors (Applied Biosystems, UK). The Prosep A slurry was added to the column and packed using the tapping method. The column was then sealed and flow packed with phosphate buffer (as described in 6.2.3.1) using a High-Performance Liquid Chromatography (HPLC) system (TOSOH Bioscience, Japan). Once prepared the column was stored at 2-8 °C with a 20 % ethanol buffer.

7.2.1.3 Affinity purification

A HPLC system was prepared with a pump (TSK 6010), an injection valve and 5 ml loop (TSK 9010), a proportioning valve (TSK 6025) and a 280 nm UV detector fluid preparation flow cell (TSK 6041). All buffers were degassed by purging with helium. The loading buffer used was the phosphate buffer (as described in 6.2.3.1). All antisera were purified using the same method and this was done sequentially. The column was flushed with at least 4 times the bed volume of loading buffer at a speed of 1

ml/minute, until a stable UV baseline was achieved. The sera were loaded via a syringe into the injection loop then processed through the column. The waste peak was observed and the column was pumped with loading buffer, until a stable UV baseline was reached again. Orthophosphoric acid at pH 2.7 was used as an elution buffer. The elution buffer was pumped through the column at 1 ml/minute until a rise in was seen in the UV reading. At this point the fraction was collected in a sterile 30 ml universal, until the peak dropped and returned to the baseline. The collected fraction was then immediately neutralised with 1.25 M NaOH, on a drop by drop basis using pH indicator strips. The elution buffer was then used to flush the column with at least 3 times the bed volume, to insure there was no residual bound material. Prior to the next loading the column was reset by flushing with at least 3 times the bed volume of loading buffer. Before storage the collected fraction was analysed using a UV spectrophotometer. The concentration of the antibody was calculated using a standard curve for IgG, the path length of the cuvette and the absorbance at 280 nm. Then each collected fraction was filtered through a syringe filter (0.2 µm) and frozen at -80 °C.

7.2.2 IMS bead coating and blocking

The frozen antibody solutions prepared in 7.2.1 were defrosted and equilibrated to room temperature.

All purified antibodies were first tested for activity against the *S*. Typhi live vaccine strain, Ty21A. This was done by suspending a 1 µl loop of biomass from a blood agar plate, in 100 µl of sterile saline on a glass slide. A drop of purified antisera was then placed on the suspension and mixed, by rocking for 2 minutes at room temperature. A negative control was included which was neutralised orthophosphoric acid, as per 7.2.1.3. Positive agglutination was scored if clumping was seen after 2 minutes. The O9, O12 and Vi purified antisera were tested separately for their ability to agglutinate with whole, live cells of Ty21A.

Coating and blocking of magnetic beads was carried out as per the methods in Chapter 6, except for two modifications. The antibodies were initially mixed in the presence of a small amount of the bovine

serum albumin (BSA) solution as described in 6.2.3.4. The BSA solution was added at 0.1 ml per gram of beads. This small addition of BSA had previously improved binding efficiencies for antibodies from purified antisera (personal observation). Also, the coating level was increased to 6 mg of antibody per gram of activated beads. This was to improve the capture efficiency of the finished IMS bead.

7.2.3 Performance testing

Performance testing of the IMS beads was carried out as specified in 6.3, with *S*. Typhi Ty21a as the marker target organisms and *E. coli* ATCC 25922 as the negative control. *E. coli* was used as the negative control, as it is a representative of the common enteric microflora expected in contaminated water. Ty21a was used as it was the only representative of *S.* Typhi available that could be handled in a containment level 2 laboratory. The *Salmonella* IMS beads described in Chapter 6 and an externally sourced anti-*Salmonella* IMS bead (Neogen, USA), were tested alongside the three IMS beads described in this chapter. All tests were performed with four replicates and recoveries calculated by comparing the mean CFU/ml of the IMS reaction, with the recovered colony counts after IMS. Columbia agar with 5 % defibrinated horse blood (LAB001, Lab M) was used as the plating media for all tests.

7.2.4 Chromogenic agar testing

Qualitative (2.3.3) and Quantitative (2.3.2) recovery of Ty21a was tested on CASE (as described in Chapter 3) Xylose Lysine Deoxycholate agar (XLD) and ABC agar (HAL001, LABM M). All three agars were challenged with an 80-120 CFU challenge and a high level (~10^6) 5µl full plate streak. All agars incubated at 37 °C for 18-24 hours.

7.2.5 Containment level 3 *S.* Typhi isolate testing

7.2.5.1 Chromogenic agar testing

The feedback from PHE indicated poorer growth on CASE compared to bismuth sulphite agar, with actual *S.* Typhi isolates. Pre-empting the cause of the suboptimal performance to be one of the

selective agents, four versions of the chromogenic agar were prepared. One was the standard formulation (as specified as the nonanoate base in Table 3.3), which was designated CASE. One had the same base but both the novobiocin and cefsulodin removed completely, which was designated CASE-. The final two had the novobiocin and cefsulodin present individually (at the same levels as the standard CASE) and were designated CASE+1 and CASE+2 respectively. ABC agar and SCA (as described in Appendix 1) were also prepared. All agars were sterilised by bringing to the boil, before cooling to 47-50 °C and pouring into Petri dishes.

All agars were tested with a panel of *S.* Typhi isolates as described in Table 7.1. These organisms represented 18 isolates implicated in bacteraemia cases of *S.* Typhi globally. The collection was provided by PHE (Colindale) as part of the Bill and Melinda Gates foundation initiative.

Table 7.1 *S.* Typhi isolate collection used to test the performance of CASE and other agars during containment level 3 testing. Each isolate is categorised by source, year and location of isolation and phage type (PT).

Sample Number	Sample Details
1	Isolate - faeces. Year - 2009. Country - Nepal. Phage type - PT E9
2	Isolate - Blood, Year - 2012, Country - Malawi, Phage type - PT E1
3	Isolate - Blood, Year - 2012, Country - Vietnam, Phage type - Untyp VI
4	Isolate - Blood. Year - 2012. Country - Congo. Phage type - PT A
5	Isolate - Blood. Year - 2013. Country - Sudan. Phage type - PT A
6	Isolate - faeces. Year - 2013. Country - Niger. Phage type - PT E1
7	Isolate - faeces. Year - 2013. Country - Nigeria. Organism - Typhi. Phage Type - Degr. VI.
8	Isolate - Blood. Year - 2014. Country - Cameroon. Phage type - PT C1.
9	Sample type - human. Isolate - faeces. Year - 2014. Country - India. Phage type - PT A
10	Isolate - Blood. Year - 2014. Country - India, Phage type - PT E9 Var
11	Isolate - blood. Year - 2014. Country - Ethiopia. Phage type - PT D1
12	Isolate - blood. Year - 2014. Country - Ghana. Phage type - PT C1
13	Isolate - U/K. Year - 2014. Country - Zimbabwe. Phage type - PT E1
14	Isolate - Blood. Year - 2015. Country - Angola. Phage type - PT E1
15	Isolate - Blood. Year - 2015. Country - Tanzania. Phage type - PT E1
16	Isolate - Blood. Year - 2015. Country - Pakistan. Phage - untype VI
17	H150 (incomplete data)
18	Isolate - Blood. Year - 2015. Country - Uganda. Phage type - PT E1

All agars were tested with Qualitative (2.3.3) recovery of all isolates described in Table 7.1. The isolates had been enriched overnight in selenite cystine broth (CM0699, Oxoid) and were streaked on the agar surface using a 5 µl loop. All agars were then incubated at 37 °C for 18-24 hours.

7.2.5.2 *S.* Typhi IMS testing

Due to the limited availability of time in the containment level 3 laboratory suite only the anti-*Salmonella* IMS beads (as described in Chapter 6) were tested, with several of the isolates in Table 7.1. These beads were selected as they had shown ability to capture Ty21a, whilst the other described in this chapter had not.

Performance testing of the IMS beads was carried out as specified in 6.3, with isolates 1, 2, 3, 8, and 11. These were selected as they represented examples of the phage types available. Each isolate was tested in duplicate. Ready-made Columbia blood agar (PB0122, Oxoid) was used as the plating medium. Decimal dilution of the overnight selenite cystine broths (as described in 7.2.5.1) were performed in phosphate buffered saline (PBS). This dilution of approximately 10^4 CFU/ml was used as the test sample. Recovery was judged qualitatively by eye as plate counts were difficult to count accurately. A decimal dilution of an overnight enrichment of *E. coli* ATCC 25922 in buffered peptone water (BPW) was used as a negative control, tested using the same IMS protocol.

7.3 Results

7.3.1 IMS bead performance

7.3.1.1 Antibody agglutination test

Prior to coating the purified antibodies were tested for their ability to bind the Ty21a strain, by slide agglutination. Figure 7.1 displays the result after 2 minutes of gentle mixing.

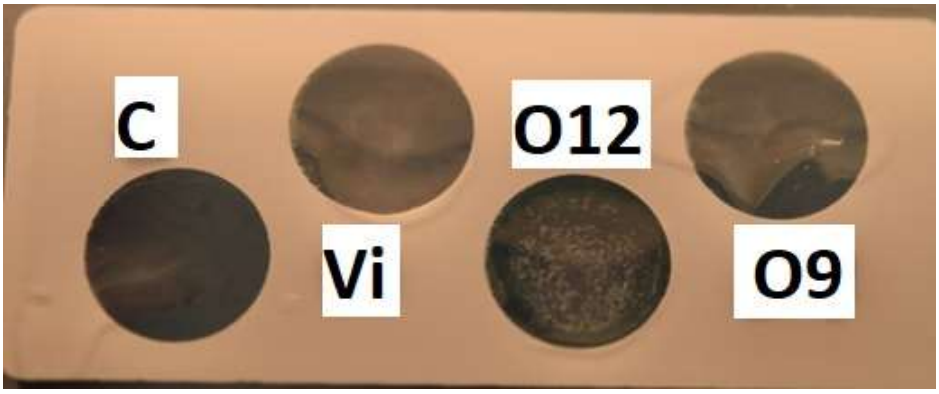


Figure 7.1 Side agglutination of the purified antibodies as described in 7.2.1.3. The reactions are labelled with the correlating test antibody and C=control.

The control showed no agglutination as expected. The Vi and O9 showed no visible agglutination after two minutes, whilst the O12 showed a strong positive result. All three purified antisera were used to produce IMS beads despite the agglutination results, as it was suspected that Ty21a was not entirely representative of *S.* Typhi. Ty21A was created by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine mutagenesis which resulted in alterations in the antigenic characteristics, such as inability to synthesize the Vi antigen (McKenna *et al*., 1995). Thus, Ty21A whilst a useful marker organism, it could not be used as a reliable representation of the typical *S.* Typhi antigenic expression.

7.3.1.2 IMS bead performance testing

All beads were tested for their ability to capture the target organism, Ty21a and the non-target *E. coli* ATCC 25922. Enumerations of the test organisms showed that Ty21a was tested at 500 CFU/ml and E. *coli* ATCC 25922 at 2067 CFU/ml. Table 7.2 displays the plate counts and calculated percentage recovery of all beads tested.

Table 7.2 Colony counts and percentage recovery of the IMS beads described in 7.2.3. Sal=IMS beads described in Chapter 6, Neo=commercially available anti-*Salmonella* beads from Neogen (USA), O9=IMS beads produced from O9 antiseria, O12=IMS beads produced from O12 antiseria, Vi=IMS beads produced from Vi antiseria.

Ty21a Post-IMS Testing	**Sal**	**Neo**	**O9**	**O12**	**Vi**
No. of colonies on plate 1.	225	225	4	7	5
No. of colonies on plate 2.	254	254	2	2	2
No. of colonies on plate 3.	267	267	4	2	1
No. of colonies on plate 4.	209	209	1	3	1
Mean colonies recovered	239	239	3	4	2
Percentage recovery	**48%**	**48%**	**1%**	**1%**	**0%**
***E.* coli ATCC 25922** **Post-IMS Testing**	**Sal**	**Neo**	**O9**	**O12**	**Vi**
No. of colonies on plate 1.	3	29	74	121	134
No. of colonies on plate 2.	2	35	59	103	131
No. of colonies on plate 3.	1	57	79	134	151
No. of colonies on plate 4.	5	88	98	164	119
Mean colonies recovered	3	52	78	131	134
Percentage recovery	**0%**	**3%**	**4%**	**6%**	**6%**

The performance testing showed that only the previously produced *Salmonella* IMS and the Neogen IMS bead successfully recovered the target organism. None of the purified antisera products successfully recovered the target. The previously produced *Salmonella* IMS bead was superior for non-target capture. The rest of the IMS products tested had low levels of non-specific binding. The binding

chemistry of the Neogen bead is unknown, but all the rest were coated using the same covalent coupling technique. Thus, it can be assumed that the non-specific binding is due to the antibody not the binding chemistry, since the generic *Salmonella* bead had almost no nonspecific capture. It is unknown if the failure of the purified antisera IMS beads was due to coating failure or Ty21a lacking the required antigenic cell surface targets.

7.3.2 Chromogenic agar performance

Ty21a was tested on XLD, ABC and CASE. All three agars gave very weak atypical growth on the full plate streaks (i.e. poor growth on the primary inoculum only, exhibiting small, colourless colonies). Quantitative testing yielded almost no recovery on all media. It was suspected that Ty21a lacks the ability to proliferate on selective agars due to its mutations. The organism can grow on non- selective Columbia blood agar (as shown 7.3.1.2), this may be due to the lack of ability to tolerate common bile acid selectivity employed in the media used.

7.3.3 Containment level 3 testing results

7.3.3.1 Chromogenic Agar

CASE, the variants with modified selective agents (as described in 7.2.5.1), ABC and SCA (Appendix 1) were tested against a panel of 18 *S.* Typhi wild type isolates. Table 7.3 displays the inoculation results.

Table 7.3 Growth response of the 18 isolates described in Table 7.1 on CASE, CASE variants (described in 7.2.5.1), ABC agar and SCA (as described in Appendix 1). Growth is graded from weak (+) to strong (+++), NG=no growth and asterisk denotes atypical colour reaction.

Sample Number	CASE	CASE-	CASE+1	CASE+2	ABC	SCA
1	+	+++	+	+++	+++	NG
2	+	+++	+	+++	+++	NG
3	+	+++	+	+++	+++	NG
4	++	+++	++	+++	+++	NG
5	+	+++	+	+++	+++	NG
6	+	+++	+	+++	+++	NG
7	+	+++	+	+++	+++*	NG
8	+++	+++	+++	+++	+++	+*
9	+	+++	+	+++	+++*	NG
10	+	+++	+	+++	+++	NG
11	++	+++	++	+++	+++	++*
12	+	+++	+	+++	+++	NG
13	++	+++	++	+++	+++	+*
14	+	+++	+	+++	+++	NG
15	+	+++	+	+++	+++	NG
16	+	+++	+	+++	+++	NG
17	+	+++	+	+++	+++	+*
18	+	+++	+	+++	+++	NG

The results replicated the initial feedback that most of the *S.* Typhi isolates exhibited a poor growth response. The standard formulation of CASE and CASE just with novobiocin (CASE+1) had the exact same poor growth response. Whilst CASE with no antibiotics (CASE-) and just cefsulodin and no novobiocin (CASE+2) have the same excellent growth response. This clearly demonstrates that the novobiocin was the cause of the poor growth response in the standard CASE formulation. Overall ABC performed well but there were two isolates, samples 7 & 9 which failed to produce a green colour reaction. This would suggest that these isolates are α-galactosidase negative and thus, cannot use the

target chromogen. SCA performed exceptionally poorly with all isolates, either failing to grow or producing weak growth with no chromogenic reaction. Figure 7.2 shows the difference between CASE with and without novobiocin, as well as a false negative on ABC agar.

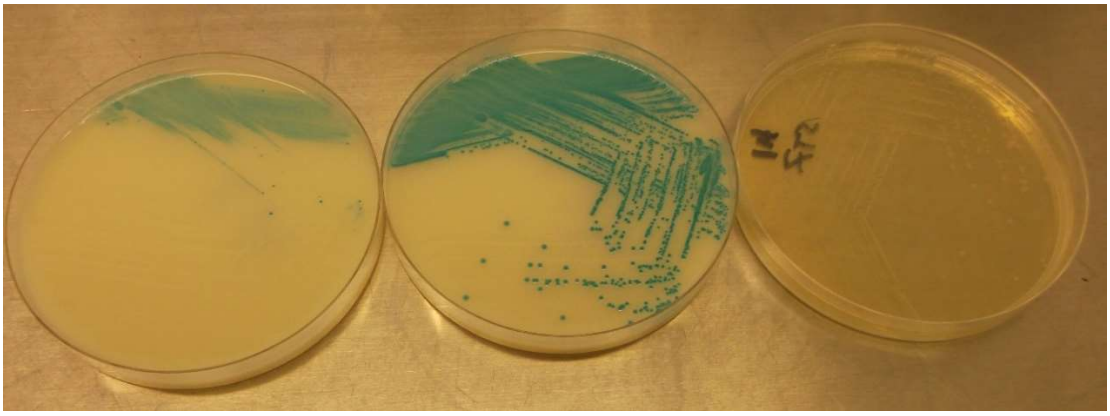


Figure 7.2 Growth response of *S.* Typhi isolate sample 7 (detailed in Table 7.1) on CASE (left), CASE without novobiocin (middle) and ABC agar (right).

7.3.3.2 IMS beads

A selection of representative *S.* Typhi isolates (1, 2, 3, 8, and 11) were tested with the *Salmonella* IMS bead described in Chapter 6. The IMS bead performed well with all isolates exhibiting a high level of capture. *E. coli* exhibited negligible recovery counts demonstrating low non-specific binding. The test demonstrated good cross reactivity with a variety of wild type *S.* Typhi isolates, suggesting the beads could be implemented in the field to capture *S.* Typhi in the environment.

7.4 Discussion

Diagnostic tests, especially microbiological methods are currently sub-optimal for use in the areas of the world greatest effected by enteric fever caused by *S.* Typhi (Baker *et al*., 2010). It is only through greater development of diagnostic tests will the burden of the pathogen be confronted. The parts of the world that are most affected by the pathogen tend to be low income areas, where high cost molecular platforms in specialist facilities are not feasible. Thus, traditional microbiology (and variants thereof) remains the most practical approach in these scenarios. Febrile disease is common in areas

affected by *S.* Typhi, meaning accurate diagnostics is critical to management of the pathogen. The cause of infection may have come from a multitude of sources, such as drinking water, contaminated food and living environment. To isolate and ultimately prevent infection of the pathogen, cultural diagnostics need to be able to handle a wide range of sample matrices.

CASE has already demonstrated its enhanced ability to detect common *Salmonella* in food (Chapters 3-5), but due to the safety restriction had not been tested with *S.* Typhi. The revelation that CASE, in its standard formulation was inhibitory to *S.* Typhi isolates was important since it is not overtly apparent in the literature. It is possible that the organism is much less tolerant to novobiocin when grown on CASE, compared to other media. This is possibly because other selective pressures (including the other antibiotic, cefsulodin) in the formulation are having a cumulative effect on *S.* Typhi. Without novobiocin the formulation supported good growth and recovery, suggesting that the inhibitory effect is synergistic. The fact that ABC agar showed atypical reactions, demonstrates that current chromogenic agars, are not necessary suitable for *S.* Typhi. CASE without novobiocin offers an improved alternative to currently available media. The selectivity lost by the omission of novobiocin should not affect the ability of the agar to detect the target organism. This is because typically enrichment in selenite cysteine broth offers excellent selection before plating. Furthermore, sample preparation with IMS would also greatly reduce the number of non-*Salmonella* inoculated on the agar.

The initial attempt to create a *S.* Typhi specific bead appears to have been unsuccessful. However, this may only be because the testing with Ty21a did not reveal their true affinity for the *S.* Typhi cell surface. Ty21a was a useful marker organism that allowed for the research to be carried out at containment level 2. This helped maximise the time available at the PHE laboratory. However, Ty21a is not representative of *S.* Typhi in terms of growth response or certain cell surface targets. This is noted by the absence of growth of Ty21a on the commercial agars tested, including ABC agar which was shown to grow wild type isolates. Further testing is required to attain if the three IMS beads produced in this chapter, can capture and concentrate wild type *S.* Typhi. It is possible that the

purification or coating was unsuccessful. It was more likely the coating, as O12 demonstrated positive agglutination after purification, meaning there was an adequate quantity of functional antibody present. It is also possible that the antigens are not suitable for effective IMS targets. To capture and concentrate an organism, the target antigen needs to be stable (strong enough to withstand the interaction and washing steps) and exposed sufficiently on a whole live cell so that capture can take place. Also, the expression of the polysaccharide antigens on the cell surface is critical for IMS capture. *S.* Typhi are known to be able to alter host immune response by changing their antigenic properties (Baker *et al*., 2010). This means that the pathogen may not strongly express a given antigen when in certain environments, like outside of the host. The generic anti-*Salmonella* bead showed good performance against the *S*. Typhi isolates tested. Even though the bead would capture other serotypes besides *S.* Typhi, it would still be a useful tool for concentrating this bacterium. Any other *Salmonella* captured and isolated would also allow for better understanding of the microflora present in the environment. Whilst *S.* Typhi and other *Salmonella enterica* serovars lead to different clinical manifestations, they share a high degree of genetic similarity (Garai *et al*., 2012). This suggests that if non-typhoidal *Salmonella* are present in the environment, there is a possibility that *S.* Typhi could also be present as it has very similar growth requirements. The data collected during the initial testing suggested that the beads described in Chapter 3 were superior to commercially available anti-*Salmonella* beads, regarding better specificity and non-specific binding. This is likely due to the binding chemistry employed, which is extremely non-reactive after blocking. This would be important to an IMS bead that would be used in raw water testing, as high background non-target organism would be present.

CASE and the generic IMS bead offer a potential workflow that would improve the ability to recover and detect *S.* Typhi. It is worth noting a key finding of this study, in that Ty21a is not a reliable representation of *S.* Typhi for cultural methodologies. The next stage of research should challenge the IMS beads and chromogenic agar with matrices that are intended to be analysed such as ground water and sewage.

Chapter Eight

Final Discussion

8.1 General discussion

Salmonella is an extraordinary successful organism which is capable of diverse infection strategies (Behnsen *et al*., 2015), can develop in a wide range of animal hosts and is able to persist in the environment (Hoszowski *et al*., 2016). The attempts to control the pathogen in Europe have largely been successful, with a large reduction in human cases between 2008 and 2016 (EFSA and ECDC, 2017). The success has been due to well implemented national control programs. An example is the national control program for *Salmonella* in the poultry sector in the UK, which has not only resulted a drop in *Salmonella* prevalence but a maintenance of low rates of incidence (O'Brien, 2013). Despite the success of control programs in Europe, *Salmonella* remains an important and high impact pathogen (Hugas and Beloeil, 2014). More recent developments regarding *Salmonella* in the food chain have seen a significant increase of new serovars, such as monophasic variants of *S.* Typhimurium which are now prevalent in the pork industry in the UK (APHA, 2016). The most important part of any surveillance initiative is accurate detection in order to inform the appropriate intervention strategy. *Salmonella* detection methodologies must be able to not only recover the pathogen, but do so in a timely manner. Also important is the ability to analyse trends and sources of the organism. By far the greatest leap forward in epidemiology has been the advent and implementation of genomics and metagenomics (Besser, 2018). Such methods, however, have their burdens and disadvantages. Public Health England (PHE) now routinely uses whole genome sequencing (WGS) for public health surveillance of *Salmonella* (Ashton *et al*., 2016). The system was implemented in April 2015 and since then there have been examples of its success, such as the detection and association of an outbreak of *S.* Enteritidis (Inns *et al*., 2017). However, this implementation took significant resources and time. Other challenges raised by the use of non-standard cultural methodologies are disruption of trend monitoring, possible misdiagnosis and loss of antimicrobial susceptibility testing (Cronquist *et al*., 2012). For these reasons cultural methods are still critical and very much at the forefront of *Salmonella* detection. Furthermore, cultural methods are a prerequisite for further analysis by methods like WGS. Chromogenic agar for *Salmonella* esterase (CASE) described in Chapter 3, presents an improvement

in diagnostic agar available for *Salmonella enterica* detection. The formulation majorly improves the ability to detect significant pathogens such as *S.* Dublin, which produces a very weak colour change on commercially available chromogenic agars. It also can recover slow growing, non-motile serovars such as *S.* Gallinarum, which is heavily supressed on many commercial chromogenic agars. Both serovars are of great importance to the food production industry and in the case of *S.* Dublin, potentially pose a threat to public health (McDonough *et al*., 1999). CASE also improves on the clarity of result, due to clear colour differentiation of target and non-target bacteria. This aids identification since the target organism is much more clearly differentiated from non-target bacteria. The green and black colours on CASE are more distinct than the purple and blue used by most commercial chromogenic agars for *Salmonella.*

Resuscitation and enrichment are the foundations of all current detection methodologies, employed to detect *Salmonella* contamination in the food chain. The ISO 6579 workflows major flaw is the time to result, taking at least three days to detect *Salmonella*. It does however have a major advantage over rapid methodologies, in that it uses dual enrichment and dual agar plating, which increases the chance of detection of low level contamination. This also can be considered a disadvantage as it consumes more resource and time. The rapid, one broth one plate (OBOP) methodology described in this study offers a major improvement in time and a reduction in resource. Since the plating medium is CASE, it means that there is improved ability to detect atypical isolates (H_2S negative or lactose positive), which may be misinterpreted on the traditional media used by ISO 6579. Like all methodologies there are limitations. As described in Chapter 4, a combination of very low-level contamination, high background microflora and interfering matrices (such as soft cheese) may result in failure of detection. It is however, worth noting that the standard methodology also struggles with this scenario. Despite these possible limitations the study described in Chapter 5, demonstrated the alternative method had comparable performance to the traditional workflow, with minced meat retail products. This reinforces the findings in Chapter 4, that most matrix types are suitable for the alternative method.

The pork and turkey mince method comparison also yielded a prevalence of *Salmonella* contamination typical of European products of this type (Little *et al*., 2008b, Boughton *et al*., 2004). At the time of writing the recovered isolates are still being serotyped, so it is not possible to compare serotypes to what is commonly isolated from these matrix types. The later part of the retail screen attempted to focus on organic and/or outside reared produce. The sample size was too low to make any meaningful comparison to indoor reared produce, but the comparison is interesting due to the potentially higher risk involved with outdoor reared animals with regards to maintenance of biosecurity (Jensen *et al*., 2006).

The immunomagnetic separation (IMS) beads described in Chapter 6, effectively captured and concentrated *Salmonella enterica* in solution, in the presence of high background microflora. The method and materials used in production yielded a cost-effective test, that could be paired with any enrichment protocol to improve the sensitivity of the methodology. The possible limitations of the IMS bead are that a very small proportion of *Salmonella enterica* serovars, would present weak affinity for the antibody used. The specific niche for this kind of technology was described in Chapter 7. By focusing the target of the beads to the specific capture of a single serovar, namely *S.* Typhi, the beads performance was greatly improved. Also, it is the detection of environmental *S.* Typhi from water samples, where the technology can be most appropriately used to deal with the problem of a large sample volume. The IMS beads can complement many different platforms of detection such as molecular based detection methods (Bakthavathsalam *et al*., 2013). They will also help deal with the issue of inhibitors which may negatively influence a polymerase chain reaction (PCR) and lead to a false result (Schrader *et al*., 2012), by reducing the influence of the matrix via washing steps. It is however, the ability to isolate and grow live *S.* Typhi cells that is more beneficial, since genetic analysis of water samples can be unreliable and not necessarily correlate with the outbreak of disease (Karkey *et al*., 2016). The modified CASE formulation allows for accurate recovery and identification of *S.* Typhi and could be pared with IMS, to increase its sensitivity. The development described in Chapter 7 was limited by the available access to a containment level 3 laboratory environment, where *S.* Typhi can

be handled. The study showed the importance of working with and testing of wild type isolates, as well as culture collection type strains to properly evaluate and improve diagnostic tests and detection methodologies.

8.1 Further Work

The greatest strength of the chromogenic agar, CASE described in Chapter 3 was the sensitivity achieved by the nonanoate ester substrate. The difference between the octanoate substrate commonly utilised and the nonanoate substrate used in CASE, was the higher affinity of some *Salmonella enterica* serovars for the nonanoate. There are several other chain lengths of esterase substrates that could be evaluated for further application. Investigations have been carried out into the use of different chain length esterase substrates (Cooke *et al*., 1999), however the results are conflicting as the study by Cook *et al.* suggests that the nonanoate should not work well for *Salmonella*. This is most likely due to the chromophore pairing, the resulting molecule and the interaction with bacterial enzymes. As CASE demonstrates the indoxyl derivative of nonanoate works exceedingly well for the detection of *S. enterica* esterase activity. So, it may be possible that other chain length indoxyl esters (or other chromophore derivatives) may achieve better differentiation of bacterial species. As the indoxyl nonanoate appears to be ideal for *Salmonella,* it may be useful to find other chromogens to prevent false negatives from organisms like *Pseudomonas* or *Aeromonas*. This way if any wild type esterase producing non-target isolates are able to overcome the selectivity of CASE, another chromogen (of different colour) would mask their nonanoate activity. Due to the possibility of cefsulodin resistant *Pseudomonas* spp. presenting false negatives on CASE, an investigation was carried out into potential masking chromogens. Studies by Laine *et al.* described the use of a β-alanyl aminopeptidase chromogen for the detection of *Pseudomonas* (Laine *et al.,* 2009). A 7-amino-4-methylocourmarin (AMC) labelled β-alanyl fluorogen was sourced to evaluate the substrate. Unfortunately, the fluorogen was also utilised by *S.* Typhimurium ATCC 14028 as well as *P.*

aeruginosa ATCC 27853 (data not shown). Further investigations could be carried out into other chromogenic/fluorogenic substrates to achieve better differentiation on a chromogenic agar.

The selectivity of both CASE and the enrichment broth (described in Chapter 4) utilise known selective compounds that are favourable for the recovery of *S. enterica.* In both instances the formulations whilst not described in the literature, employ variations of selective tactics used by researchers for many years. Novel antimicrobial strategies could be employed to yield better selectivity than currently available. The modification of CASE (as described in Appendix 1) effectively used sucralose as a selective agent. The synthetic organochlorine sweetener is widely researched but the biological interactions of the compound is relatively understudied (Schiffman and Rother, 2013). It is possible that the molecule acts as a 'metabolic distraction', as sucrose fermenting organisms may waste energy trying to metabolise the compound, which is unavailable due to the chlorination. The compound also seems to have inherent bacteriostatic effects on some bacteria. There is significant study into the effect of artificial sweeteners on the microbiome of humans (Suez *et al*., 2015), as the compounds are ingested as a sugar replacement. However, there is little to no literature on the utilisation of the bacteriostatic or bactericidal properties of the compounds in microbiological culture media. These findings suggest the possibility of utilising the compound and other similar compounds (artificial sweeteners) in selective culture media.

Work regarding improved *S.* Typhi detection methods is ongoing as the project is aiming to start field trials in Malawi soon. Current planned work in this area is the development of further IMS beads. Dr Andrew Jackson of the University of Liverpool is part of the same *S.* Typhi project (as described in Chapter 7) funded by The Bill & Melinda Gates Foundation. Dr Jacksons group is examining the possibility that environmental contamination of *S.* Typhi may be residing in *Acanthamoebae*, a single-celled eukaryote. The increased persistence of *S.* Typhi in the environment in the presence of *Acanthamoebae* has been previously described (Douesnard-Malo and Daigle, 2011). The intention is to develop an IMS bead with monoclonal antibodies raised against *Acanthamoebae*, to use as a tool

to help determine if environmental samples contain *S.* Typhi hidden in this environmental host. This may be more complicated than a bacterial IMS bead (like the *Salmonella enterica* bead developed in Chapter 6), as a eukaryote capture protocol may require bead-organism dissociation for further study. Currently considered is the use of acid or heat dissociation, dependant on if the removal of the bead is critical to the *S.* Typhi detection method.

8.1 Final Conclusions

Detection methodologies are critical for the proper execution and maintenance of *Salmonella* control measures, to help reduce prevalence in the food chain and incidence of infection. This study has yielded a novel chromogenic agar formulation for *Salmonella enterica* detection, with superior sensitivity and specificity compared to currently available diagnostic agar formulations. A faster, single step enrichment method has been paired with the novel agar, to achieve a faster time to result for many food matrices types. IMS technology was utilised to aid detection of *Salmonella,* with the ultimate focus of facilitating capture and concentration of *S.* Typhi from water samples. *S*. Typhi is a neglected tropical pathogen, with little development in detection methodologies being carried out for over 20 years. The modified version of the chromogenic agar and the IMS bead offers a new detection method, that yields live cells available for further study. Improved detection methods inevitably lead to a reduction of infection from this important pathogen.

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Appendix 1

CASE variation for the highly selective detection and enumeration of *S.* Typhimurium from chicken caeca.

A modification of CASE (as described in Chapter 3) was produced for the purpose *Salmonella* Typhimurium detection by the direct inoculation of chicken caeca. Caeca are part of the gastrointestinal tract of chicken, and presence and enumeration of artificially spiked *S.* Typhimurium (designation 4/74) was being investigated by Dr Lizeth Lacharme-Lora, a postdoctoral research associate at the University of Liverpool. The project is not concerned with the detection of all *Salmonella* that may be present but just the specific artificially spiked *S.* Typhimurium. The matrix material (caeca) had an exceptionally high bioburden and as such was a challenge for diagnostic agars when inoculated without any selective enrichment. The agar formulations (including CASE, other chromogenics and traditional media like xylose lactose Tergitol™ 4 agar (XLT-4)) previously used all suffered from overgrowth of non-target organisms, which obscured any *Salmonella* present. An agar was needed that could identify *Salmonella* but was selective enough to inhibit a very high bioburden of enteric organisms.

CASE is formulated to be highly selective but also to have high sensitivity to be able to detect all *Salmonella enterica* serovars. Some *Salmonella* are sensitive to certain levels of the employed selective agents, meaning that there is a compromise between selectivity and sensitivity, in favour of the later. As such there are several non-target *Enterobacteriaceae* which can grow well on CASE. However, CASE is still an effective diagnostic because it is designed to be used after selective enrichment.

The base formulation of CASE was modified to incorporate 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-gal) and 3-4-cyclohexenoesculetin-β-D-galactoside (CHE-gal). These allow the agar to differentiate *Salmonella* from other *Enterobacteriaceae* by their ability to produce α-galactosidase in the absence of β-galactosidase. This results in blue/green *Salmonella* and black non-target organism. This chromogen pairing is used in ABC agar (Perry *et al*., 1999) but is flawed in that it can fail to detect lactose positive *Salmonella*. For this application, this is not an issue because the target organism is a *S*. Typhimurium isolate which does not possess β-galactosidase. α-galactosidase

production is less effected by high selectivity than esterase production (personal observation). IPTG was added to induce the production of β-galactosidase. The bile acid content was increased to that which is used in CASE. Bile salts No. 3 was replaced with specific ratio of the individual bile acids used in bile salts No. 3, but at a higher level than that in CASE. The level of Ox bile was also doubled. Cefsulodin was lowered because *Pseudomonas* (whilst still inhibited on this formulation) is less important to inhibit because it lacks the ability to use either chromogens. Kaolin was also removed as it wasn't deemed necessary for this application.

The final formulation was as described in table A1.1 and was given the designation SCA (Leahurst).

Table A1.1 Formulation of Salmonella chromogenic agar (Leahurst formulation)

Compound	g/l
Beef Extract	2
Pork Heart Infusion	1
Meat Peptone	5
Vitamin Mix	1
Sodium Pyruvate	0.5
Tri Sodium Citrate	8.5
Sodium Deoxycholate	2.48
Sodium Cholate	2.03
Bacteriological Ox Bile	2
Disodium Hydrogen Phosphate	5.6
Potassium Dihydrogen Phosphate	1.4
Ferric Ammonium Citrate	0.5
Novobiocin	0.015
Cefsulodin	0.003
Agar No 2	12.5
IPTG	0.03
CHE-β-D-Galactoside	0.3
X-α-Galactoside	0.1

All components were weighed out to produce 3 kg of dehydrated culture media (DCM). All components except the agar were processed through a Retsch ZM 200 ultra-centrifugal mill with a

0.5mm mill screen, to reduce all particles to the same size. The Agar was then added, and all components were mixed in a 5 kg barrel for 20 minutes on a barrel roller.

It was hypothesised that sucralose could be added as a further selective pressure on non-target organisms. The concept of sucralose was that it could be a metabolic distraction for sucrose fermenting organisms. *Salmonella* do not ferment sucrose, and sucrose fermenters cannot ferment sucralose because it is trichloronated and unable to be acted upon by the same enzymes that sucrose is. It has a similar structure however, so the hypothesis would be that the sucrose fermenting organisms would produce enzymes to metabolise the compound.

An experiment was carried out where four variations of the formulation (as described in Table A1.1) were prepared. The first had no extra additions, the second had 5 g/l sucralose added, the third had 5 g/l sucrose added and the fourth had 5 g/l sodium chloride added. All additions were added before sterilisation and all media was sterilised by bring to the boil, cooled to 48 °C in a water bath and poured into Petri dishes by hand.

S. Typhimurium ATCC 14028 and *E. aerogenes* ATCC 13048 were grown overnight in tryptone soy broth (TSB) at 37 °C for 18 hours. Both organisms were then diluted in maximum recovery diluent (MRD) so that 5 µl contained approximately 10^5 CFU. These culture dilutions were individually streaked onto the agar surface of all versions of the agar, and the plates were incubated for 18-24 hours at 37 °C.

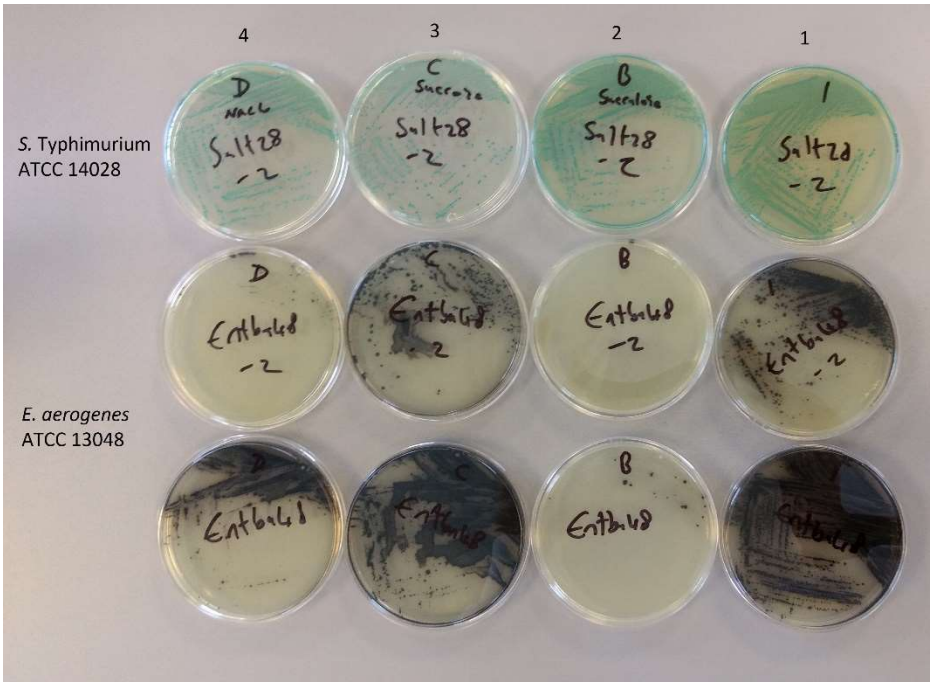


Figure A1.1 The growth of *S.* Typhimurium and *E. aerogenes* after 18 hours on SCA (Leahurst)formulation. Top row is inoculated with *S.* Typhimurium ATCC 14028 (~10^5 CFU), resulting in green colonies. Middle row is inoculated with *E. aerogenes* ATCC 13048 (~10^5 CFU), resulting in black colonies. Bottom row is inoculated with *E. aerogenes* ATCC 13048 straight from the overnight culture in TSB (~10^7 CFU), resulting in black colonies. Formulation 1 is as described in Table A1.1. Formulation 2 is the same as 1 but with the addition of 5 g/l sucralose. Formulation 3 is the same as 1 but with the addition of 5 g/l sucrose. Formulation 4 is the same as 1 but with the addition of 5 g/l sodium chloride.

As Figure A1.1 shows, all variations support the growth and identification of *S.* Typhimurium ATCC 14028. All plates yield good growth of green colonies indicating production of α-galactosidase in the absence of β-galactosidase. Without any additions, the chromogenic base (formulation 1) results in growth of *E. aerogenes* ATCC 13048 at both concentrations. The addition of sucrose (formulation 3) shows very similar growth responses to no additions. Both sucralose (formulation 2) and sodium chloride (formulation 4) show excellent suppression of *E. aerogenes*, with sucralose achieving near total inhibition of ~10^7 CFU.

All the additions create a certain level of osmotic stress to organisms growing on the media. Sodium chloride creates ionic stress whilst sucrose and sucralose cause non-ionic stress (Cheung *et al*., 2009). However, it is clearly not just osmotic stress which is responsible for the increase in selectivity observed when sucralose is added. Sucralose offers selective pressure against various microorganisms (Omran *et al*., 2013). It is likely other organisms (possibly including some *Salmonella*) would be supressed / inhibited by the sucralose, however *S.* Typhimurium ATCC 14028 appears not to be. Based on this results sucralose was incorporated into the SCA (Leahurst) formulation.

The medium was provided to Dr Lacharme-Lora at the University of Liverpool, Leahurst campus and used in the caeca study. Feedback from the study was positive, with the media achieving greater selectivity than any other media previously tests (including XLT-4). *S.* Typhimurium 4/74 grew well and produced green colonies so was easily detected on the agar.

Sucralose shows potential as a selective agent in bacterial culture media. Currently there are no described formulation utilising this compound. Other artificial sweeteners (such as saccharin and aspartame) may also be beneficial for microbial culture media.

Appendix 2

Formulations of Buffered Peptone Water (BPW) and selective supplements described in chapter 2

Table A2.1 Formulation of BPW according to ISO 6579-1:2017.

Component	g/l
Peptone	10
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.2 Formulation of aBPW described in chapter 2.

Component	g/l
Yeast Extract	7
Soy Peptone	1.5
Casein Peptone	1.5
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.3 Formulation of LAB204 described in chapter 2.

Component	g/l
Tryptone	10
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.4 Formulation of LAB046 described in chapter 2.

Component	g/l
Meat Peptone	5
Tryptone	5
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.5 Formulation of C3M3S3V1 described in chapter 2.

Component	g/l
Casein Peptone	3
Meat Peptone	3
Soy Peptone	3
Vitamin Mix	1
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.6 Formulation of C4M5Y1 described in chapter 2.

Component	g/l
Casein Peptone	4
Meat Peptone	5
Yeast Extract	1
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.7 Formulation of C5Y5 described in chapter 2.

Component	g/l
Casein Peptone	5
Yeast Extract	5
Sodium Chloride	5
Disodium Hydrogen Phosphate (anhydrous)	3.57
Potassium Dihydrogen Phosphate	1.5

Table A2.8 Formulation of CNSX supplement described in chapter 2.

Component	mg/l
Cefsulodin	3
Novobiocin	20
Sodium Sulfadiazine	80
Xylose	1250

Table A2.9 Formulation of NSM supplement described in chapter 2.

Component	mg/l
Novobiocin	25
Sodium Sulfadiazine	40
Mannitol	500

Appendix 3

Full table of results from chapter 4, 4.4.4 Temperature Experiment

Table A3.1 Results of all samples tested in both Lab M and Acumedia BPW at two temperatures and multiple time points, as described in the temperature experiment (4.2.3). Growth is graded by +=primary inoculum growth, ++=growth up to second quadrant, +++=growth up to third quadrant, NG=no growth. Colour of colonies stated after growth rating. Organism short codes designated in Table 3.1 & 3.2.

	Acumedia BPW (aBPW) incubated at 37 °C		
	16 h	**18 h**	**24 h**
L1	+++ Green		
L2	+++ Green		
L3	+++ Green		
L4	+++ Green		
L5	+++ Green		
L6	+++ Green (Pale)		
L7	+++ Green		
L8	+++ Green		
L9	+++ Green (Pale)		
L10	+++ Green		
L11	+++ Green		
L12	+++ Green		
L13	+++ Green		
L14	+++ Green		
L15	+++ Green		
Salpo40	+++ Green		
Salt28	++ Green		
Salv77	+++ Green		
Salar55	+++ Green		
Sale76	++ Green		
Sald74	+++ Green (Pale)		
Sale76 +Entba48	++ Black	++ Black	++ Black
Sale76 +Ec22	++ Colourless	++ Colourless	++ Colourless
Shso74	+++ Colourless	+++ Colourless	+++ Colourless
Shso30	+++ Colourless	+++ Colourless	+++ Colourless
Prom83	NG	NG	NG
Prom38	NG	NG	NG
Ec22	+++ Colourless	+++ Colourless	+++ Colourless
Ec39	20pp Colourless	10pp Colourless	20pp Colourless
Psa53	+++ Blue	+++ Blue	+++ Blue
Psa27	4 Blue	20 Blue	+++ Blue
Entba48	+++ Black	+++ Black	+++ Black

	Acumedia BPW (aBPW) incubated at 41.5 °C		
	16 h	**18 h**	**24 h**
L1	+++ Green		
L2	+++ Green		
L3	+++ Green		
L4	+++ Green		
L5	+++ Green		
L6	+++ Green (Pale)		
L7	+++ Green		
L8	+++ Green		
L9	+++ Green (Pale)		
L10	+++ Green		
L11	+++ Green		
L12	+++ Green		
L13	+++ Green		
L14	+++ Green		
L15	+++ Green		
Salpo40	+++ Green		
Salt28	++ Green		
Salv77	+++ Green		
Salar55	+++ Green		
Sale76	++ Green		
Sald74	+++ Green		
Sale76 +Entba48	+++ Green +Black	+++ Green +Black	+++ Green +Black
Sale76 +Ec22	++ Green +Colourless	++ Green +Colourless	+++ Green +Colourless
Shso74	++ Colourless	++ Colourless	++ Colourless
Shso30	+++ Colourless	+++ Colourless	+++ Colourless
Prom83	NG	NG	NG
Prom38	NG	NG	NG
Ec22	+++ Colourless	+++ Colourless	+++ Colourless
Ec39	+++ Colourless	+++ Colourless	+++ Colourless
Psa53	NG	NG	NG
Psa27	NG	NG	NG
Entba48	+++ Black	+++ Black	+++ Black

	Lab M BPW (LAB204) incubated at 37 °C		
	16 h	**18 h**	**24 h**
L1	++ Green		
L2	+++ Green		
L3	+++ Green		
L4	+ Green		
L5	+++ Green		
L6	++ Green (Pale)		
L7	+ Green		
L8	+++ Green		
L9	++ Green (Pale)		
L10	++ Green		
L11	++ Green		
L12	++ Green		
L13	+++ Green		
L14	+++ Green		
L15	+++ Green		
Salpo40	+++ Green		
Salt28	++ Green		
Salv77	++ Green		
Salar55	+++ Green		
Sale76	++ Green		
Sald74	+ Green		
Sale76 +Entba48	++ Green +Black	++ Green +Black	++ Green +Black
Sale76 +Ec22	++ Green +Colourless	+++ Green +Colourless	+++ Green +Colourless
Shso74	+ Colourless	+ Colourless	++ Colourless
Shso30	+ Colourless	+ Colourless	+ Colourless
Prom83	NG	NG	NG
Prom38	NG	NG	NG
Ec22	NG	NG	+ Colourless
Ec39	NG	NG	+ Colourless
Psa53	NG	NG	NG
Psa27	NG	NG	NG
Entba48	++ Black	++ Black	++ Black

	Lab M BPW (LAB204) incubated at 41.5 °C		
	16 h	**18 h**	**24 h**
L1	++ Green		
L2	+++ Green		
L3	+++ Green		
L4	+ Green		
L5	+++ Green		
L6	++ Green (Pale)		
L7	+ Green		
L8	+++ Green		
L9	+ Green (Pale)		
L10	+ Green		
L11	+ Green		
L12	++ Green		
L13	+++ Green		
L14	+++ Green		
L15	+++ Green		
Salpo40	++ Green		
Salt28	++ Green		
Salv77	++ Green		
Salar55	+++ Green		
Sale76	++ Green		
Sald74	+ Green		
Sale76 +Entba48	++ Green +Black	++ Green +Black	++ Green +Black
Sale76 +Ec22	++ Green	+++ Green	+++ Green
Shso74	NG	+ Colourless	+ Colourless
Shso30	NG	NG	+ Colourless
Prom83	NG	NG	NG
Prom38	NG	NG	NG
Ec22	NG	NG	NG
Ec39	NG	NG	NG
Psa53	NG	NG	NG
Psa27	NG	NG	NG
Entba48	+ Black	+ Black	++ Black

Appendix 4

Full table of results from Chapter 4, 4.4.6 Matrices Experiment

Table A4.1 Results of spiked matrix testing on CASE and XLD, with and without secondary enrichment in RVS as described in the matrices experiment (4.2.6). Refer to table key for result codes.

Table Key	
m+?	Positive, minority of colonies, small/weak colonies
m+	Positive, minority of colonies
M+	Positive, majority of colonies
NEG	Negative
-	No result recorded

Matrices	Replicate Number	LAB204 (X)				C4M5V1 (Y)				C5Y5 (Z)			
		CASE	RVS>CASE	XLD	RVS>XLD	CASE	RVS>CASE	XLD	RVS>XLD	CASE	RVS>CASE	XLD	RVS>XLD
Raw (unpasteurised) Milk + ~10 CFU Sale76	1	m+?	M+	M+	M+	m+?	M+	M+	M+	m+?	M+	M+	M+
	2	m+?	M+	M+	m+	m+?	M+	M+	M+	m+?	M+	M+	M+
	3	m+?	M+	M+	m+	m+?	M+	M+	M+	m+?	M+	M+	M+
	4	m+?	M+	NEG	m+	m+?	M+	M+	M+	m+?	M+	M+	M+
	5	m+?	M+	NEG	m+	m+?	M+	NEG	M+	m+?	M+	NEG	M+
	6	m+?	M+	NEG	m+	m+?	M+	NEG	M+	m+?	M+	NEG	M+
	7	m+?	M+	NEG	m+	m+?	M+	NEG	m+	m+?	M+	NEG	M+
	8	m+?	M+	NEG	m+	m+?	M+	NEG	m+	m+?	M+	NEG	m+
	9	m+?	m+	NEG	m+	m+?	M+	NEG	m+	m+?	m+	NEG	m+
	10	m+?	NEG	NEG	NEG	m+?	m+	NEG	m+	m+?	-	NEG	m+
	Sum	0+	9+	3+	9+	0+	10+	4+	10+	0+	9+	4+	10+
Chicken skin + ~10 CFU Sale76	1	m+	NEG	m+	M+	m+	NEG	m+	NEG	m+	m+	m+	m+
	2	m+	M+	m+	M+	NEG	m+	m+	NEG	m+	m+	m+	m+?
	3	m+	m+	m+	m+	NEG	m+	NEG	m+?	m+	m+?	m+	m+
	4	m+	M+	NEG	M+	NEG	NEG	NEG	NEG	m+	M+	NEG	m+
	5	m+	M+	NEG	NEG	NEG	NEG	NEG	m+?	m+	M+	NEG	m+
	6	m+	M+	NEG	M+	NEG	NEG	NEG	NEG	m+	m+?	NEG	m+?
	7	m+	M+	NEG	M+	NEG	m+?	NEG	m+?	m+	m+?	NEG	m+?
	8	NEG	NEG	NEG	m+	NEG	M+	NEG	m+	NEG	m+?	NEG	m+
	9	NEG	M+	NEG	M+	NEG	m+?	NEG	NEG	NEG	m+?	NEG	m+
	10	NEG	NEG	NEG	m+?	NEG	NEG	NEG	NEG	NEG	m+?	NEG	m+
	Sum	7+	7+	3+	8+	1+	3+	2+	1+	7+	4+	3+	7+

Matrices	Replicate Number	LAB204 (X)				C4M5V1 (Y)				C5Y5 (Z)			
		CASE	RVS>CASE	XLD	RVS>XLD	CASE	RVS>CASE	XLD	RVS>XLD	CASE	RVS>CASE	XLD	RVS>XLD
Unpasteurised soft cheese (Brie) + ~10 CFU Sale76	1	m+	M+	m+	M+	M+	M+	m+	m+	m+	M+	m+	m+
	2	m+	M+	m+	m+	m+	M+	m+	m+	m+	M+	m+	m+
	3	m+	m+	m+	m+	m+	M+	m+	m+	NEG	m+	NEG	m+
	4	NEG	m+	NEG	m+	m+	M+	NEG	m+	NEG	m+	NEG	NEG
	5	NEG	NEG	NEG	NEG	NEG	m+	NEG	NEG	NEG	NEG	NEG	NEG
	6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	9	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	10	-	NEG	NEG	NEG	NEG	-	NEG	-	NEG	NEG	NEG	NEG
	Sum	3+	4+	3+	4+	4+	5+	3+	4+	2+	4+	2+	3+
Pork mince (high fat) + ~10 CFU Sale76	1	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	2	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	3	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	4	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	5	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	6	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+	M+
	7	M+	M+	M+	M+	M+	M+	m+	M+	M+	M+	M+	M+
	8	M+	M+	M+	M+	M+	m+	m+	M+	M+	M+	M+	M+
	9	M+	M+	M+	M+	m+	m+	m+	M+	M+	M+	M+	M+
	10	M+	m+	M+	M+	m+	m+	NEG	m+	m+	M+	m+	M+
	Sum	10+	10+	10+	10+	10+	10+	9+	10+	10+	10+	10+	10+
Organic leafy greens (salad leaves) + ~10 CFU Sale76	1	m+	M+	m+	M+	m+	m+	NEG	m+	m+	M+	m+	m+
	2	m+	M+	m+	m+	m+	m+?	NEG	m+	m+	M+	NEG	m+
	3	m+	m+?	m+	m+?	m+	M+	NEG	M+	m+	NEG	NEG	m+?
	4	m+	M+	NEG	m+	m+	m+	NEG	m+	m+	M+	NEG	m+?
	5	m+	M+	NEG	m+?	m+	M+	NEG	m+	NEG	M+	NEG	m+?
	6	m+	M+	NEG	m+	NEG	M+	NEG	m+	NEG	M+	NEG	m+
	7	m+	M+	NEG	m+	NEG	m+?	NEG	m+	NEG	M+	NEG	m+
	8	m+	M+	NEG	m+	NEG	m+	NEG	M+	NEG	M+	NEG	m+
	9	m+	M+	NEG	m+	NEG	M+	NEG	m+	NEG	M+	NEG	m+
	10	NEG	M+	NEG	m+	NEG	M+	NEG	m+	NEG	M+	NEG	m+
	Sum	9+	9+	3+	8+	5+	8+	0+	10+	4+	9+	1+	7+

Appendix 5

Full table of results from Chapter 5, Retail produce testing.

Table A5.1 Pork mince information and testing results from the first round of testing as described in Chapter 5. +=positive results, -=negative result. Latex confirmation only carried out on presumptive positive results.

				ISO 6579 Method				Final Result	Alternative Method BPW+ → Plate	Latex	Final Result
Sample	Source	Origin	Batch Information	XLD	Latex	CASE	Latex		CASE		
1	1	UK	UK 2458 EC 7133408 02:21 BMKC	-		-		-	-		-
2	1	UK	UK 2458 EC 7133408 02:21 BMKC	+	-	-		-	-		-
3	1	UK	UK 2458 EC 7133408 02:21 BMKC	+	-	-		-	-		-
4	1	UK	UK 2458 EC 7133408 02:21 BMKC	+	-	-		-	-		-
5	1	UK	UK 2458 EC 7133408 02:21 BMKC	+	-	-		-	-		-
6	1	UK	UK 2458 EC 7133408 02:21 BMKC	+	-	-		-	-		-
7	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
8	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
9	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
10	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
11	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
12	1	UK	UK 2358 EC 6135408 02:22 LK/AK	+	-	-		-	-		-
13	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
14	1	UK	UK 2358 EC 6135408 02:22 LK/AK	-		-		-	-		-
15	1	UK	UK 2458 EC 7133408 02:21 BMKC	-		-		-	-		-
16	2	UK	D5 471 140 08:18	+	-	-		-	-		-
17	2	UK	D5 471 142 12.25	+	-	-		-	-		-
18	2	UK	D5 471 142 12.25	+	-	-		-	-		-
19	2	UK	D5 471 138 08:55	-		-		-	-		-
20	2	UK	D5 471 139 07:21	+	-	-		-	-		-
21	2	UK	D5 471 139 07:21	-		-		-	-		-
22	2	UK	D5 707 142 18:18	-		-		-	-		-
23	2	UK	D5 707 142 18:18	+	-	-		-	-		-

24	2	UK	D5 707 142 18:18	+	-	-		-	-		-
25	2	UK	D5 705 143 11:53	+	-	-		-	-		-
26	2	UK	D5 705 142 10:22	-		-		-	-		-
27	2	UK	D5 705 142 10:22	-		-		-	-		-
28	2	UK	D5 705 142 10:22	+	-	-		-	-		-
29	2	UK	D5 705 142 10:22	-		-		-	-		-
30	2	UK	D5 705 143 11:53	-		-		-	-		-
31	1	UK	UK 2458 EC 9188107 10:00 DK/RJ	-		-		-	-		-
32	1	UK	UK 2458 EC 9188107 10:00 DK/RJ	-		-		-	-		-
33	1	UK	UK 2458 EC 9188107 10:00 DK/RJ	-		-		-	-		-
34	1	UK	UK 2458 EC 9188107 10:00 DK/RJ	-		-		-	-		-
35	1	UK	UK 2458 EC 8187407 22:53 DJ/EJ	-		-		-	-		-
36	1	UK	UK 2458 EC 8187407 22:53 DJ/EJ	+	-	-		-	-		-
37	3	UK	UK 4787 EC L9 231 10:20	+	-	-		-	-		-
38	3	UK	UK 4787 EC L9 227 06:34	-		-		-	-		-
39	3	UK	UK 4787 EC L9 231 10:20	-		-		-	-		-
40	3	UK	UK 4787 EC L9 231 10:20	-		-		-	-		-
41	3	UK	UK 4787 EC L9 231 10:20	-		-		-	-		-
42	3	UK	UK 4787 EC L9 227 06:34	-		-		-	-		-
43	3	UK	UK 4787 EC L9 231 10:20	+	-	-		-	-		-
44	3	UK	UK 4787 EC L9 231 13:00	-		-		-	-		-
45	3	UK	UK 4787 EC L9 231 13:00	-		-		-	-		-
46	3	UK	UK 4787 EC L9 231 13:00	-		-		-	-		-
47	3	UK	UK 4787 EC L9 231 13:00	-		-		-	-		-
48	3	UK	UK 4787 EC L9 231 13:00	-		-		-	-		-
49	3	UK	UK 4787 EC L9 231 10:20	-		-		-	-		-
50	3	UK	UK 4787 EC L9 227 06:34	-		-		-	-		-
51	3	UK	UK 4787 EC L9 231 10:20	-		-		-	-		-
52	3	UK	UK 4787 EC L9 231 13:00	+	-	-		-	-		-
53	3	UK	UK 4787 EC L9 231 13:00	+	-	-		-	-		-

Table A5.2 Fruit and vegetable information and testing results from the second round of testing as described in Chapter 5. +=positive results, -=negative result. Latex confirmation only carried out on presumptive positive results.

				ISO 6579 Method				Result	Alternative Method	Result
Sample	Description	Source	Origin	XLD	Latex	CASE	Latex		CASE	
1	Salad Tomatoes	1	Netherlands	-		-		-	-	-
2	Salad Tomatoes	1	Netherlands	-		-		-	-	-
3	Salad Tomatoes	1	Netherlands	-		-		-	-	-
4	Salad Tomatoes	1	Netherlands	-		-		-	-	-
5	Whole Cucumber	1	Netherlands	-		-		-	-	-
6	Whole Cucumber	1	Netherlands	-		-		-	-	-
7	Whole Cucumber	1	Netherlands	-		-		-	-	-
8	Unwashed Sliced Curly Kale	1	UK	-		-		-	-	-
9	Unwashed Sliced Curly Kale	1	UK	+	-	-		-	-	-
10	Unwashed Sliced Curly Kale	1	UK	-		-		-	-	-
11	Unwashed Sliced Curly Kale	1	UK	-		-		-	-	-
12	Washed Baby Spinach	1	UK	-		-		-	-	-
13	Washed Baby Spinach	1	UK	-		-		-	-	-
14	Washed Baby Spinach	1	UK	+	-	-		-	-	-
15	Honeydew Melon	1	NOT STATED	-		-		-	-	-
16	Honeydew Melon	1	NOT STATED	-		-		-	-	-
17	Honeydew Melon	1	NOT STATED	-		-		-	-	-
18	Galia Melon	1	NOT STATED	-		-		-	-	-
19	Galia Melon	1	NOT STATED	-		-		-	-	-
20	Galia Melon	1	NOT STATED	-		-		-	-	-
21	Cantaloupe	3	SPAIN	-		-		-	-	-
22	Cantaloupe	3	SPAIN	-		-		-	-	-
23	Cantaloupe	3	SPAIN	-		-		-	-	-
24	Cantaloupe	3	SPAIN	-		-		-	-	-
25	Cantaloupe	3	SPAIN	-		-		-	-	-
26	Honeydew Melon	3	SPAIN	-		-		-	-	-
27	Honeydew Melon	3	SPAIN	-		-		-	-	-
28	Honeydew Melon	3	SPAIN	-		-		-	-	-
29	Honeydew Melon	3	SPAIN	+	-	-		-	-	-
30	Honeydew Melon	3	SPAIN	-		-		-	-	-

Table A5.3 Minced meat information and testing results from the final round of testing as described in Chapter 5. ✓=positive results, ×=negative result, ?=data unknown. Green highlight indicates confirmed positive via latex agglutination.

Product	Origin	% Fat	Batch	Expiry Date	Outdoor?	Organic?	ISO	Alternative
Pork Mince	4	10	?	24.03.17	No	No	×	×
Pork Mince	4	10	?	25.03.17	No	No	×	×
Pork Mince	4	10	?	25.03.17	No	No	×	×
Pork Mince	4	10	?	24.03.17	No	No	×	×
Pork Mince	4	10	?	25.03.17	No	No	×	×
Pork Mince	4	10	?	24.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	27.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	29.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	29.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	29.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	28.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	28.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	27.03.17	No	No	×	×
Pork Mince	5	5	UK 2060 EC	29.03.17	No	No	×	×
Turkey Mince	1	7	UK 7013 EC	26.03.17	No	No	×	×
Pork Mince	1	20	UK 2458 EC	28.03.17	No	No	×	×
Turkey Mince	1	7	UK 7013 EC	26.03.17	No	No	×	×
Pork Mince	1	20	UK 2458 EC	28.03.17	No	No	×	×
Pork Mince	1	20	UK 2458 EC	28.03.17	No	No	×	×
Turkey Mince	5	7	UK 5049 EC	24.03.17	No	No	×	×
Turkey Mince	5	7	UK 5049 EC	27.03.17	No	No	×	×
Turkey Mince	5	7	UK 5049 EC	27.03.17	No	No	×	×
Pork Mince (Outdoor)	6	7	?	22.03.17	Yes	No	×	×
Pork Mince (Outdoor)	6	7	?	26.03.17	Yes	No	×	×
Pork Mince (Outdoor)	6	7	?	22.03.17	Yes	No	×	×
Pork Sausage	5	9	KT032	01.04.17	No	No	×	×
Turkey Mince	5	7	UK 5049 EC	27.03.17	No	No	×	×
Pork Sausage	5	9	KT032	03.04.17	No	No	×	×
Turkey Mince	4	5	5049	24.3.17	No	No	✓	✓
Turkey Mince	4	5	2013	27.03.17	No	No	×	✓
Turkey Thigh Mince	4	7	4013	27.03.17	No	No	×	×
Turkey Mince	5	7	5049	24.03.17	No	No	×	×
Turkey Mince	5	7	5049	24.03.17	No	No	×	×
Pork Sausage	5	11	KT032	24.03.17	No	No	×	✓
Cumberland Pork Sausage	5	9	KT032	02.04.17	No	No	×	×
Pork Sausage	5	9	KT032	30.03.17	No	No	×	×
Pork Sausage	6	10	WZ014	26.03.17	No	No	×	×
Pork Sausage	6	10	WZ014	26.03.17	No	No	×	×

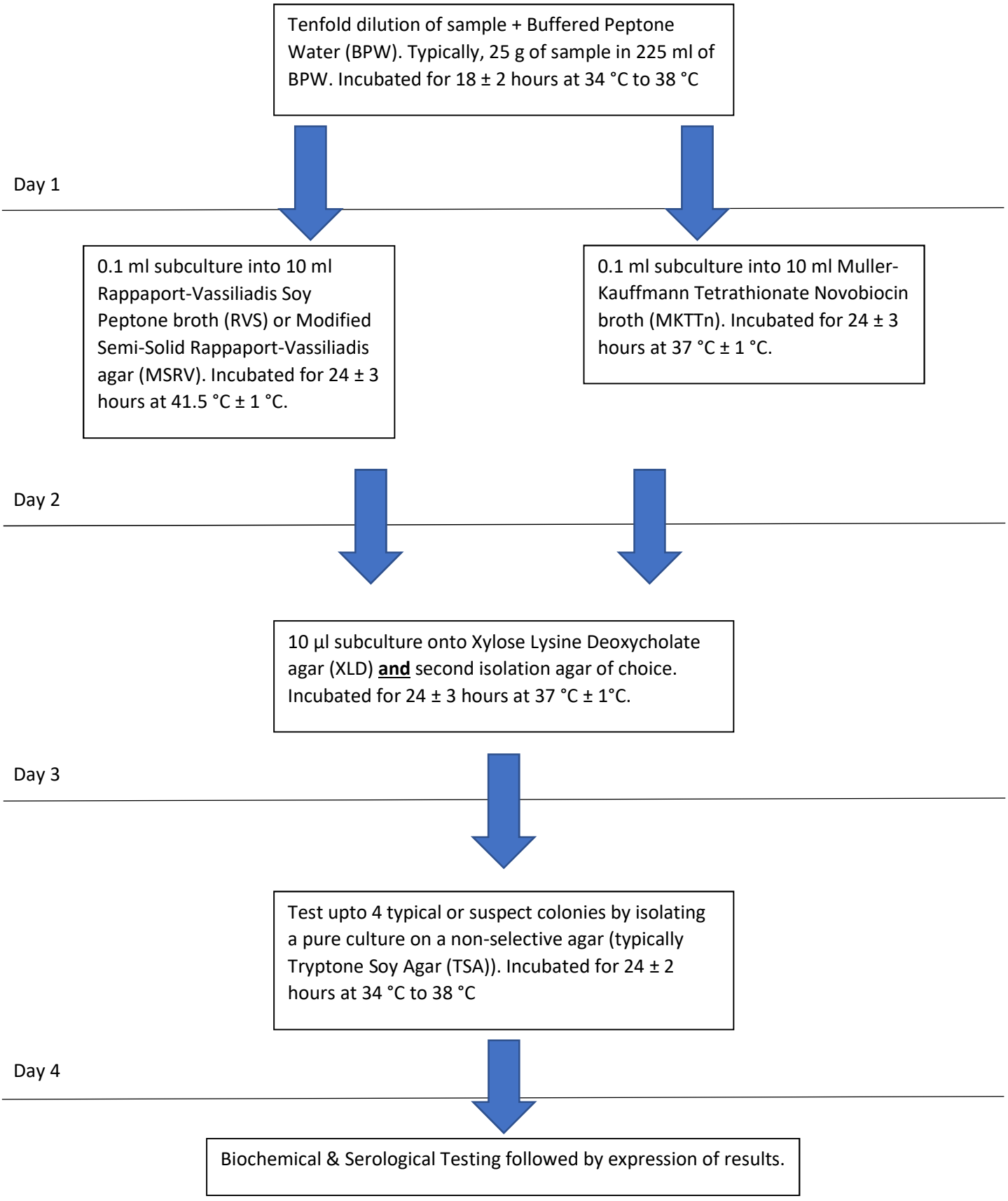
Pork Sausage	7	11	KT032	03.04.17	No	No	×	×
Pork Sausage	5	11	KT032	28.03.17	No	No	×	×
Pork Mince	5	5	2060	29.03.17	No	No	×	✓
Pork Mince	5	5	2060	25.03.17	No	No	×	×
Pork Mince	5	5	2060	30.03.17	No	No	×	×
Pork Sausage	7	8	KT032	03.04.17	No	No	×	×
Pork Mince	8	x	4707	07.04.17	Yes	Yes	×	×
Pork Mince	8	x	4707	07.04.17	Yes	Yes	×	×
Pork Mince	8	x	4707	07.04.17	Yes	Yes	×	×
Pork Mince	9	8	8367	06.04.17	Yes	Yes	×	×
Pork Mince	9	8	8367	06.04.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	07.04.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	07.04.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	07.04.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	07.04.17	Yes	Yes	×	×
Pork Mince	11	10	IE 409 EC	07.04.17	No	No	×	×
Pork Mince	11	10	IE 409 EC	07.04.17	No	No	×	×
Free Range Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Free Range Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Free Range Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Pork Mince	13	8	5242	06.04.17	Yes	Yes	×	×
Pork Mince	13	8	5242	06.04.17	Yes	Yes	×	×
Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Pork Mince	12	8	5242	06.04.17	Yes	?	×	×
Turkey Mince	12	7	9509	08.04.17	?	?	✓	×
Turkey Mince	12	7	9509	08.04.17	?	?	×	×
Turkey Mince	12	7	9509	08.04.17	?	?	×	×
Turkey Mince	11	2	5337	08.04.17	?	?	×	×
Turkey Mince	11	2	5337	08.04.17	?	?	×	×
Turkey Mince	11	2	5337	08.04.17	?	?	×	×
Pork Sausage	12	~25	5077	09.04.17	Yes	?	×	×
Cumberland Pork Sausage	12	~25	5077	08.04.17	Yes	?	×	×
Pork Chipolatas	12	~15	5077	10.04.17	Yes	?	×	×
Pork Chipolatas	11	?	C1D08	06.04.17	?	?	×	×
Turkey Breast Mince	12	2	5004	30.07.17	Yes	?	×	×
Turkey Breast Mince	12	2	5004	30.07.17	Yes	?	×	×
Turkey Breast Mince	12	2	5004	30.07.17	Yes	?	×	×
Turkey Thigh Mince	12	8	5004	30.07.17	Yes	?	×	×
Turkey Thigh Mince	12	8	5004	30.07.17	Yes	?	×	×
Turkey Thigh Mince	12	8	5004	30.07.17	Yes	?	×	✓
Turkey Thigh Mince	11	6	5337	29.07.17	?	?	×	×
Turkey Thigh Mince	11	6	5337	29.07.17	?	?	×	×

Turkey Thigh Mince	11	6	5337	29.07.17	?	?	✓	✓
Turkey Thigh Mince	11	6	5337	29.07.17	?	?	✓	✓
Pork Mince	11	10	409	30.07.17	?	?	×	×
Pork Mince	11	10	409	30.07.17	?	?	×	×
Pork Mince	6	7	2093	30.07.17	Yes	?	×	×
Pork Mince	6	7	2093	30.07.17	Yes	?	×	×
Free Range Pork Mince	12	8	5242	28.07.17	Yes	?	×	×
Free Range Pork Mince	12	8	5242	28.07.17	Yes	?	×	×
Pork Mince	9	8	8367	29.07.17	Yes	Yes	×	×
Pork Mince	9	8	8367	29.07.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	28.07.17	Yes	Yes	×	×
Pork Mince	10	<30	1040	28.07.17	Yes	Yes	×	×
Sausage meat	12	~20	5077	31.07.17	Yes	?	×	×
Sausage meat	12	~20	5077	31.07.17	Yes	?	×	×
Irish Sausages	14	~20	557	06.07.17	?	?	×	×
Irish Sausages	14	~20	557	06.07.17	?	?	×	×
Pork Sausage	6	~20	JF062	02.08.17	?	?	×	×
Chipolata Pork Sausage	6	12	JF062	30.07.17	?	?	×	×
Pork Sausage	6	~20	JF062	02.08.17	?	?	×	×
Pork Chipolatas	11	?	C1D09	27.07.17	?	?	×	×
Pork Sausage	11	?	C1D09	27.07.17	?	?	×	×
Pork Mince	4	5	UK 2093 EC	10.09.17	?	?	✓	✓
Pork Mince	4	5	UK 2093 EC	10.09.17	?	?	×	×
Pork Mince	4	5	UK 2093 EC	10.09.17	?	?	✓	✓
Pork Mince	4	5	UK 2093 EC	10.09.17	?	?	×	×
Pork Mince	4	5	UK 2093 EC	10.09.17	?	?	×	×
Pork Mince	4	10	UK 2060 EC	09.09.17	?	?	×	×
Pork Mince	4	10	UK 2060 EC	09.09.17	?	?	×	×
Pork Mince	4	10	UK 2060 EC	09.09.17	?	?	×	×
Pork Mince	4	10	UK 2060 EC	09.09.17	?	?	×	×
Pork Mince	4	10	UK 2060 EC	09.09.17	?	?	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Pork Mince (Organic)	4	10	UK 2093 EC	09.09.17	Yes	Yes	×	×
Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×
Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×
Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×
Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×

Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×
Turkey Breast Mince	4	2	UK 7013 EC	11.09.17	?	?	×	×
Turkey Thigh Mince	4	7	UK 7013 EC	11.09.17	?	?	×	×
Turkey Thigh Mince	4	7	UK 7013 EC	11.09.17	?	?	×	×
Turkey Thigh Mince	4	7	UK 7013 EC	11.09.17	?	?	×	×
Turkey Thigh Mince	4	7	UK 7013 EC	11.09.17	?	?	×	×
Turkey Thigh Mince	4	7	UK 7013 EC	11.09.17	?	?	×	×
Pork Sausage meat	4	26	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Sausage meat	4	26	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Sausage meat	4	26	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Sausage meat	4	26	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Sausage meat	4	26	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Sausage	4	36	UK HU 200 EC	08.09.17	Yes	Yes	×	×
Pork Sausage	4	36	UK HU 200 EC	08.09.17	Yes	Yes	×	×
Pork Sausage	4	36	UK HU 200 EC	08.09.17	Yes	Yes	×	×
Pork Sausage	4	36	UK HU 200 EC	08.09.17	Yes	Yes	×	×
Pork Cocktail Sausages	4	10	UK HU 200 EC	06.09.17	Yes	?	×	×
Pork Cocktail Sausages	4	10	UK HU 200 EC	06.09.17	Yes	?	×	×
Pork Cocktail Sausages	4	10	UK HU 200 EC	06.09.17	Yes	?	×	×
Pork Cocktail Sausages	4	10	UK HU 200 EC	06.09.17	Yes	?	×	×
Pork Chipolatas	4	15	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Chipolatas	4	15	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Chipolatas	4	15	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Chipolatas	4	15	UK HU 200 EC	11.09.17	Yes	?	×	×
Pork Chipolatas	4	15	UK HU 200 EC	11.09.17	Yes	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×
Turkey Sausages	4	8	UK 7013 EC	08.09.17	?	?	×	×

Appendix 6

Workflow diagram of ISO 6579-1:2017 standard detection protocol for food.


Tenfold dilution of sample + Buffered Peptone Water (BPW). Typically, 25 g of sample in 225 ml of BPW. Incubated for 18 ± 2 hours at 34 °C to 38 °C
Day 1
0.1 ml subculture into 10 ml Rappaport-Vassiliadis Soy Peptone broth (RVS) or Modified Semi-Solid Rappaport-Vassiliadis agar (MSRV). Incubated for 24 ± 3 hours at 41.5 °C ± 1 °C.
0.1 ml subculture into 10 ml Muller-Kauffmann Tetrathionate Novobiocin broth (MKTTn). Incubated for 24 ± 3 hours at 37 °C ± 1 °C.
Day 2
10 µl subculture onto Xylose Lysine Deoxycholate agar (XLD) and second isolation agar of choice. Incubated for 24 ± 3 hours at 37 °C ± 1°C.
Day 3
Test upto 4 typical or suspect colonies by isolating a pure culture on a non-selective agar (typically Tryptone Soy Agar (TSA)). Incubated for 24 ± 2 hours at 34 °C to 38 °C
Day 4
Biochemical & Serological Testing followed by expression of results.
Day 5